Psyche

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Volume 2012
Part IV

Psyche: A Journal of Entomology

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Volume 2012, Part IV

ISSN: 0033-2615 (Print), ISSN: 1687-7438 (Online), DOI: 10.1155/6152

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 532652, 14 pages
doi:10.1155/2012/532652

Research Article

Pheromone Production, Attraction, and Interspecific Inhibition among Four Species of *Ips* Bark Beetles in the Southeastern USA

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Received 9 December 2011; Accepted 26 January 2012

Academic Editor: John A. Byers

Hindgut volatiles from attacking, unmated males of *Ips avulsus, I. calligraphus, I. grandicollis*, and *I. pini* were analyzed by combined gas chromatography and mass spectrometry. Based on the quantitative identifications of hindguts and subsequent individual aerations, baits were formulated and a combined species-specific subtractive field bioassay was set up for the four bark beetle species. The bioassays were subtractive for the compounds identified in the hindgut analysis of each species, and volatiles identified in sympatric species were added as potential inhibitors alone and in combination. The trap catches from this bioassay revealed strong interspecific inhibition. The subtractive assays showed that *I. grandicollis* and *I. calligraphus* share (−)-(4*S*)-*cis*-verbenol as one pheromone component, while their second, synergistic pheromone component, (−)-(*S*)-ipsenol in *I. grandicollis* and (±)-ipsdienol in *I. calligraphus*, acts as an interspecific inhibitor to the other species. *I. avulsus* and *I. pini* were found to have very similar production of hindgut volatiles, and both use ipsdienol and lanierone as synergistic pheromone components. No beetle-produced interspecific inhibitor was identified between these two species. Lanierone was found to be an interspecific inhibitor for both *I. calligraphus* and *I. grandicollis*.

1. Introduction

The bark beetle genus of *Ips* is circumpolar, and different species range all over the northern hemisphere. In North America, there are 25 species of *Ips* [1], and of these, four species are indigenous to the Southeastern USA [2–4]. *Ips calligraphus* (Germar) has its range predominantly in the coastal plain, *I. pini* (Say) is limited to the Appalachian mountain range, while *I. avulsus* (Eichhoff) and *I. grandicollis* (Eichhoff) both have a wider range and are found in the entire area. The ranges of *I. calligraphus* and *I. pini* never overlap, while *I. avulsus* and *I. grandicollis* are sympatric with both *I. calligraphus* and *I. pini*. All four species utilize several species of pine (*Pinus*) as their host trees, but there is a slight difference in host preferences. Eastern white pine (*P. strobus* L.) is the major host tree for *I. pini* in the Southeastern USA, while the other three *Ips* species below the mountain range use several species of pine, such as loblolly pine (*P. taeda* L.), shortleaf pine (*P. echinata* Miller), slash pine (*P. elliotii* Engelmann), longleaf pine (*P. palustris* Miller), and Virginia pine (*P. virginiana* Muller).

Bark beetles, like many insects, utilize semiochemicals to find a mate and to concentrate their attacks on suitable host plants [5–7]. As a widespread genus, *Ips* spp. beetles were among the first to be investigated for their pheromones [8]. Vité et al. [9] found that many *Ips* species produced ipsenol, ipsdienol, or *cis*-verbenol as their aggregation pheromones either alone or in combination. At this time, a single compound was often regarded as the entire pheromone of a species [10, 11]. Subsequently, the pheromones of several bark beetle species have been reinvestigated, and new compounds have been identified and shown to be synergistic pheromone components of the aggregation pheromone blends [12–14], increasing trap catches up to more than 25 times. All these reinvestigations clearly showed that bark beetle aggregation pheromones very rarely are found to be

a single component; instead most are based on two or more beetle-produced compounds.

The male-produced pheromone compounds that were initially identified in the four *Ips* species in the southeastern USA were ipsdienol in *I. avulsus*, *I. calligraphus*, and *I. pini*; ipsenol in *I. grandicollis*; and *cis*-verbenol in *I. calligraphus* [9]. Based on range overlap in these species, the three identified compounds will not provide sufficient adequate species isolation on a pheromone basis. The aim of our study was to reinvestigate for the presence of new, male-produced pheromone candidates for all the four species and test them in subtractive trap bioassays in the field. In addition, we tested interspecific attraction and inhibition, the latter being common among sympatric bark beetle species.

2. Materials and Methods

2.1. Biological Material. Beetles were collected as brood in host pine logs and brought in to the laboratory. *Ips avulsus*, *I. calligraphus*, and *I. grandicollis* were all collected in forests around Athens, Georgia, while beetles of *I. pini* were collected in the Nanthahala National Forest, North Carolina. The emerging broods were then allowed to attack fresh pine bolts in the laboratory and kept at ambient temperature and humidity. Georgia beetles were established on loblolly pine, while *I. pini* was reared on Eastern white pine. The logs were predrilled with holes (∅ 2.5 mm) to spread the attacks over the log surface. The attacking *I. avulsus*, *I. calligraphus*, and *I. grandicollis* beetles were cut out of the bark after 36 hours, and single males in nuptial chambers were stored on dry ice until dissection and chemical analysis.

The *I. pini* beetles were collected during August and needed a diapause to produce pheromones. Without this diapause, the male beetles did not produce any pheromone components. In order to mimic a natural diapause, the beetles were allowed to attack a cut bolt of Eastern white pine, and the wood was put in a cold storage (5°C, 80% RH) for three weeks. The beetles were then removed from the bolt and reintroduced on new bolts of Eastern white pine. The reattacking beetles were cut out of their host tree after 48 and 96 hours and kept on dry ice until dissection and chemical analysis.

In order to estimate the pheromone release rates during the second day after the initiation of the attack, beetle attacks were also individually induced to provide sites for aerations. These males were put in predrilled holes in the bark (∅ 2.5 mm) and covered with gelatin capsules (No. 000; Eli Lilly & Co., Indianapolis, IN, USA) that fit into a groove made by a cork borer (∅ 9.5 mm) centered over an attack hole. The gelatin capsule prevented the males from escaping during the initiation of the attack. During the aeration, the gelatin capsule was removed and a cut piece of a Pasteur pipette was fitted over the entrance hole and was held firmly to the bark with a rubber band. A Teflon tube (55 mm × ∅ 3 mm) filled with Porapak Q (65 mg, mesh 60–80; Supelco, Bellefonte, PA, USA) fitted in the constraints of the Pasteur pipette, modified from Birgersson and Bergström [15]. Airflow of 50 mL/min was achieved with battery-operated pumps (Gilian HFS 513A; Gilian Instrument Corp., West Caldwell, NJ, USA), and collections continued for 3 hours. Similar aerations were also collected in the field from individual attacks, to compare the release rates of pheromone in the laboratory with those in the field. Aeration columns were kept in Nalgene cryogenic vials (Nalge Nunc Internat., Rochester, NY, USA) on dry ice or in a –83°C freezer until extraction and chemical analysis.

2.2. Chemical Analysis. Beetles kept on dry ice were allowed to thaw and immediately the hindgut was dissected, using a pair of sharp forceps, and transferred to a 0.3 mL Reacti-Vial (Pierce Chem. Comp., Rockford, IL, USA) chilled on dry ice, according to Birgersson et al. [16]. The sex of each beetle was absolutely determined at dissection, by the presence of aedeagus or spermatheca. Extracts were made in batches of 8 to 20 male beetles in 10 μL of redistilled pentane with 10 ng/μL of heptyl acetate (C_7Ac) for extracts of *I. avulsus* and *I. grandicollis*, and 100 ng/μL of C_7Ac for extracts of *I. calligraphus* and *I. pini*, giving 100 ng and 1000 ng of C_7Ac, respectively, as a quantification standard. The hindgut extracts were concentrated to less than 5 μL before the chemical analysis.

2.3. Aerations. The aeration columns were allowed to equilibrate at room temperature and were then eluted with 500 μL diethyl ether into tapered vial inserts (Agilent Technologies), and 1000 ng of C_7Ac was added to each extract as a quantification standard. The extracts were allowed to concentrate in a fume hood to the volume of around 20 μL prior to chemical analysis.

2.4. Chemical Identification and Quantification. All chemical analyses were performed on a combined gas chromatograph and mass spectrometer (GC-MS): Hewlett-Packard (HP) 5890 GC and a HP 5970 MS (nowadays Agilent Technologies, Palo Alto, CA, USA). The GC was equipped with either a 30 m × 0.25 mm fused silica column coated with HP-1 (100% methyl siloxane, df = 0.5 μm: Agilent Technologies) or a 50 m × 0.25 mm fused silica column, coated with HP-FFAP (nitroterphthalic acid modified polyethylene glycol, df = 0.5 μm; Agilent Technologies). Temperature programming was 50°C for 5 minutes, 8°C/min to 225°C, followed by isothermal at 225°C for 10 minutes for both columns. Injector temperature was 200°C and the transfer line was kept at 225°C. Helium was used as mobile phase, at 35 cm/s, and the electron impact (EI mode) mass spectra were obtained at 70 eV. All samples, 2 μL each, were injected manually and splitless for 0.5 minutes. Compounds were identified by their GC retentions times and obtained mass spectra and compared with authentic samples of synthetic references. Mass spectra were also compared to both commercially available MS libraries (NBS and NIST) and to our own MS database.

For chemical analyses two different fused silica columns were used, each with stationary phases of different polarity, to avoid the possible coelution of unknown compounds rendering detection, and identification difficult. Compounds that might coelute on a nonpolar stationary phase, such

Table 1: Compounds used in field bioassays: abbreviation, purity, and source.

Compound	Abbreviation	Purity	Purchased from
2-methyl-3-buten-2-ol	MB	98%	Aldrich Chemical Company Inc., Milwaukee, WI, USA
(±)-ipsdienol	Id	99%	PheroTech Inc., Delta, BC, Canada
(±)-ipsenol	Ie	99%	PheroTech Inc., Delta, BC, Canada
E-myrcenol	EM	99%	Dr. W. Francke, Universität Hamburg, Hamburg, Germany
Geraniol	Ger	99%	Aldrich Inc., Milwaukee, WI, USA
Lanierone	Ln	98%	Dr. H.-E. Högberg, Mid-Swedish University, Sundsvall, Sweden
(−)-(4*S*)-*cis*-verbenol	cV	99%	Aldrich, Milwaukee, WI, USA

as HP-1, most often will separate on a medium polar to polar stationary phase, like HP-FFAP, and *vice versa*. The dual analyses did not show any new compounds on either column. During the manual analysis of the obtained GC-MS chromatograms (HP Standalone data analysis workstation), every single peak in every analysis was checked, in order to identify and confirm the identity of new compounds. In addition, the extracted ion current profiles (EICPs) [17] allowed us to search for compounds identified in the analyses of one species, in the analytical run of another species. The use of EICP also lowered the limit of detection for the compounds to far below 1 ng injected on the GC-MS, and even less per analyzed beetle. This thorough search for new compounds probably allowed us to identify all ecologically relevant compounds in the hindgut extracts. Compounds present in one single analysis, and never found in other analyses, were defined as contaminants, since several analyses were made on each species on each column. Control analyses of extracting solvents also helped us to rule out contaminants.

Quantification of the identified compounds in the analyses was based on standard curves of selected oxygenated monoterpenes. The standard curve covered four orders of magnitude, from 1 ng to 10 μg. For identified compounds, not included in the standard curve, their response factors were assumed to be similar to related compounds in the standard mixture. In aeration analyses, with hundreds of compounds from the host trees, the quantifications were based on prominent MS fragments in the selected male beetle-produced compounds and in the quantification standard, according to Garland and Powell [17] and Dobson [18]. This method gives extracted ion current profiles (EICPs), which increases the signal-to-noise ratio tenfolds, depending on which MS fragment extracted. The EICP-method also allowed us to search for compounds identified from one analysis, in all the other analyses. As controls, both the hindgut extract solvent and the aeration extracting solvent were analyzed for contaminants.

Chiral analyses of ipsdienol, ipsenol, and *cis*-verbenol were made without derivatization of pooled remains of hindgut extract on an HP 5890A GC-FID equipped with a fused silica column (30 m × 0.25 mm) coated with methylated β-cyclodextrin (df = 0.25 μm; cylodex-B J&W Scientific Inc., Folsom, CA, USA), according to König et al. [19]. Injector and detector temperatures were 175°C and 225°C, respectively, and column temperature was held constant at 125°C, with N_2 as carrier gas at 15 cm/s. All samples were injected manually, 2 μL each, and injected splitless for 0.1 minutes.

2.5. Field Bioassays. Multiple subtractive field bioassays [20] were carried out, and the compounds used in these bioassays were selected based on the hindgut analyses made on the attacking males of each species. Compounds identified in sympatric species were added as tentative inhibitors. Release rates of the selected compounds were based on the individual entrance hole aeration analyses, made both from laboratory introduced and naturally attacking bark beetles. The release rates of the baits corresponded to the estimated amounts similar to 500–1000 male attacks on a host tree. All compounds used in the bioassays are listed in Table 1.

The compounds of each bait were mixed and dissolved in nonane and released at the listed hourly rate through 5 cm × 1.5 mm Teflon tubing, lined with a cotton yarn wick, inserted through a hole drilled in the screw top of a 2 mL glass vial [21]. These "wick-baits" were attached to the middle of 12 unit Lindgren multifunnel traps (Phero Tech Inc., Delta, BC, Canada) with binder-clips. The traps within each set were separated by at least 8 meters, twice the distance between attacked and nonattacked trees in the area. Several sets of traps, separated by at least 50 meters, were used in each bioassay. The trapsets were installed on recent clear-cuts within the range of each bark beetle species. The traps were emptied and randomized regularly, either several times a day when any trapping bait had ≥50 beetles, or on a weekly basis, depending on the population density.

The first bioassays, made in July 1990, comprised four "species-specific" pheromone blends and a subtractive assay of six volatiles identified in male hindgut extracts (Table 2). Traps were set up in Francis Marion National Forest, South Carolina, to trap *I. avulsus*, *I. calligraphus*, and *I. grandicollis*, and in Nantahala National Forest, North Carolina, to trap *I. pini*. The results from the subtractive part of this assay clearly showed very strong interspecific inhibition, and that further

Table 2: Pilot study, Francis Marion National Forest, SC, and Nantahala National Forest, NC, July 1990: pheromone candidates released from Wick-baits: for abbreviations, see Table 1. Release rates are given in μg/h.

	MB	Id	Ie	EM	Ger	cV
avulsus-bait	20	20	—	—	—	2.5
calligraphus-bait	—	100	—	—	5.0	50
grandicollis-bait	—	1.0	50	5.0	2.5	2.5
pini-bait	—	250	—	20	10	20
Subtractive assay						
Total blend (TB)	20	250	50	20	10	50
TB-MB	—	250	50	20	10	50
TB-Id	20	—	50	20	10	50
TB-Ie	20	250	—	20	10	50
TB-EM	20	250	50	—	10	50
TB-Ger	20	250	50	20	—	50
TB-cV	20	250	50	20	10	—
Blank	—	—	—	—	—	—

bioassays had to be based on species-specific subtractive assays, with addition of possible interspecific inhibitors.

The total set of the *I. grandicollis* subtractive and additive assay (Table 3) was performed in Oconee National Forest, GA, April 1992. The full blend for *I. avulsus* (Table 3) was bioassayed at Fort Benning, AL, September 1991, in conjunction with the bioassay of *I. calligraphus* (Table 3), but at different sites. The bioassay for *I. pini* (Table 3) was conducted in Rabun Co, GA, June and July 1992, and was checked and randomized on a weekly basis.

2.6. Statistical Design. Completely randomized, Latin square designs were used to position the traps in each field bioassay. Male and female beetle responses were analyzed separately, with the exception of *I. avulsus* trapped on *I. pini* bait (too many beetles trapped for sexing). Trap catches, as percentage catch to each bait in each replicate, were analyzed by ANOVA as square root $(X + 0.5)$ and arcsin square root $(\%X)$ transformations. Treatment means were separated using the Fisher's protected LSD option at $\alpha = 0.01$ when the entry F-statistic was significant at the 0.05 level. All analyses were performed using SAS-PC (SAS-Institute, Carey, NC).

3. Results

3.1. Chemical Analyses. In total, 15 bark beetle-produced, oxygenated compounds were identified and quantified in hindgut extracts from unmated males of the four *Ips* species investigated (Table 4, Figure 1). All the species had either ipsdienol or ipsenol and *cis*-verbenol, which are the most common pheromone components in the genus [9]. *E*-myrcenol was identified in *I. grandicollis* and *I. pini* male hindguts. In addition, geraniol was identified in all species. Lanierone was identified in *I. pini* and *I. avulsus*, and 2-methyl-3-buten-2-ol was identified in *I. avulsus*. Several oxygenated monoterpenes, regarded as detoxification products of host tree monoterpene hydrocarbons, were identified in all species, especially in *I. calligraphus* and *I. pini*. 2-Phenyl ethanol was identified in all species.

The males of *I. pini* were in preparation for their overwintering diapause, when they were collected in August, and produced only trace amounts of oxygenated monoterpenes when put on new logs, of their host tree, Eastern white pine (Figure 2(a)). After the cold stratification, they still did not produce any pheromone components when placed on new logs, but their production of host related oxygenated monoterpenes had increased substantially (Figure 2(b)). This cohort of beetles, when excised and put on new logs did not produce any pheromone for the first days. After 48 hours in new logs they still only contained the oxygenated monoterpenes, related to host tree resin (Figure 2(c), Table 4). However, after 96 hours following cold treatment, they produced the pheromone components ipsdienol and lanierone in large amounts. At this time only trace amounts of host tree-related, oxygenated monoterpenes were detected (Figure 2(d), Table 4).

Chiral analyses were made on ipsdienol, ipsenol, and *cis*-verbenol. All species produced (−)-(*S*)-*cis*-verbenol. Ipsenol in *I. grandicollis* was enantiomerically pure, with 100% (−)-(*S*)-isomer, while the enantiomeric compositions of ipsdienol varied widely among the three species (Table 5). The aerations of male beetles in nuptial chambers showed that the average hourly release of pheromone components closely approximates the average hindgut amounts (Table 4). Based on these results, the field bioassays were set up to release the amount of each compound equal to 500–1000 male attacks.

3.2. Field Bioassays. The results from the pilot bioassay clearly showed that there were too much interspecific inhibitions to do subtractive bioassays of all the identified compounds in one assay. This pilot study was followed by species-specific subtractive bioassays, with compounds identified in sympatric species added as tentative inhibitors.

When ipsenol was subtracted in the *I. grandicollis* subtractive assay, the trap catches was reduced to that of the blank, clearly indicating ipsenol as the key pheromone component in this species (Figure 3(a)). When *cis*-verbenol was omitted, the trap catches of both males and females dropped significantly, but not as much as when ipsenol was excluded. The trap catch of females was significantly lower when *E*-myrcenol was subtracted from the total blend, while the males were not significantly affected. Geraniol does not seem to affect the attraction of either sex. The addition of ipsdienol showed an inhibition to *I. grandicollis*. Lanierone added alone also has a significant negative impact on the trap catches. When both ipsdienol and lanierone were added in combination to the full *I. grandicollis* blend, trap catches were significantly lower, and especially so for males, where the number of beetles trapped was as low as the blank.

Table 3: Subtractive bioassay: pheromone candidates released from Wick-baits:—for abbreviations, see Table 1. Release rates are given in μg/h.

Ips grandicollis bait, used in Oconee National Forest, GA, April 1992.						
	Ie	EM	Ger	cV	Id	Ln
Total blend	250	65	65	250	—	—
– ” – – Ie	—	65	65	250	—	—
– ” – – EM	250	—	65	250	—	—
– ” – – Ger	250	65	—	250	—	—
– ” – – cV	250	65	65	—	—	—
– ” – + Id	250	65	65	250	500	—
– ” – + Ln	250	65	65	250	—	65
– ” – + Id + Ln	250	65	65	250	500	65
Blank	—	—	—	—	—	—
Ips avulsus bait, used at Fort Benning, AL, September, 1991.						
	MB	Id	Ln	cV	Ie	EM
Total blend	500	500	65	250	—	—
– ” – – MB	—	500	65	250	—	—
– ” – – Id	500	—	65	250	—	—
– ” – – Ln	500	500	—	250	—	—
– ” – – cV	500	500	65	—	—	—
– ” – + Ie	500	500	65	250	250	—
– ” – + EM	500	500	65	250	—	65
– ” – + Ie + EM	500	500	65	250	250	65
Blank	—	—	—	—	—	—
Ips calligraphus bait, used at Fort Benning, AL, September 1991.						
	Id	Ger	cV	Ie	EM	Ln
Total blend	500	65	250	—	—	—
– ” – – Id	—	65	250	—	—	—
– ” – – Ger	500	—	250	—	—	—
– ” – – cV	500	65	—	—	—	—
– ” – + Ie	500	65	250	250	—	—
– ” – + EM	500	65	250	—	65	—
– ” – + Ln	500	65	250	—	—	65
– ” – + Ie + EM + Ln	500	65	250	250	65	65
Blank	—	—	—	—	—	—
Ips pini bait, used in Rabun county, GA, June and July 1992.						
	Id	EM	Ger	Ln	cV	Ie
Total blend	500	65	65	65	250	—
– ” – – Id	—	65	65	65	250	—
– ” – – EM	500	—	65	65	250	—
– ” – – Ger	500	65	—	65	250	—
– ” – – Ln	500	65	65	—	250	—
– ” – – cV	500	65	65	65	—	—
– ” – + Ie	500	65	65	65	250	250
Blank	—	—	—	—	—	

There was no attraction by *I. avulsus* to the *I. grandicollis* blend. When ipsdienol was added, there was a weak attraction, while addition of lanierone alone showed no attraction to *I. avulsus*. However, when both ipsdienol and lanierone were added to the full *I. grandicollis* blend, the trap catches of *I. avulsus* peaked (Figure 3(b)). The number of *I. calligraphus* trapped in the bioassay with *I. grandicollis* baits was too low for statistical analysis, even though most *I. calligraphus* were trapped when ipsdienol was added alone to the *I. grandicollis* blend.

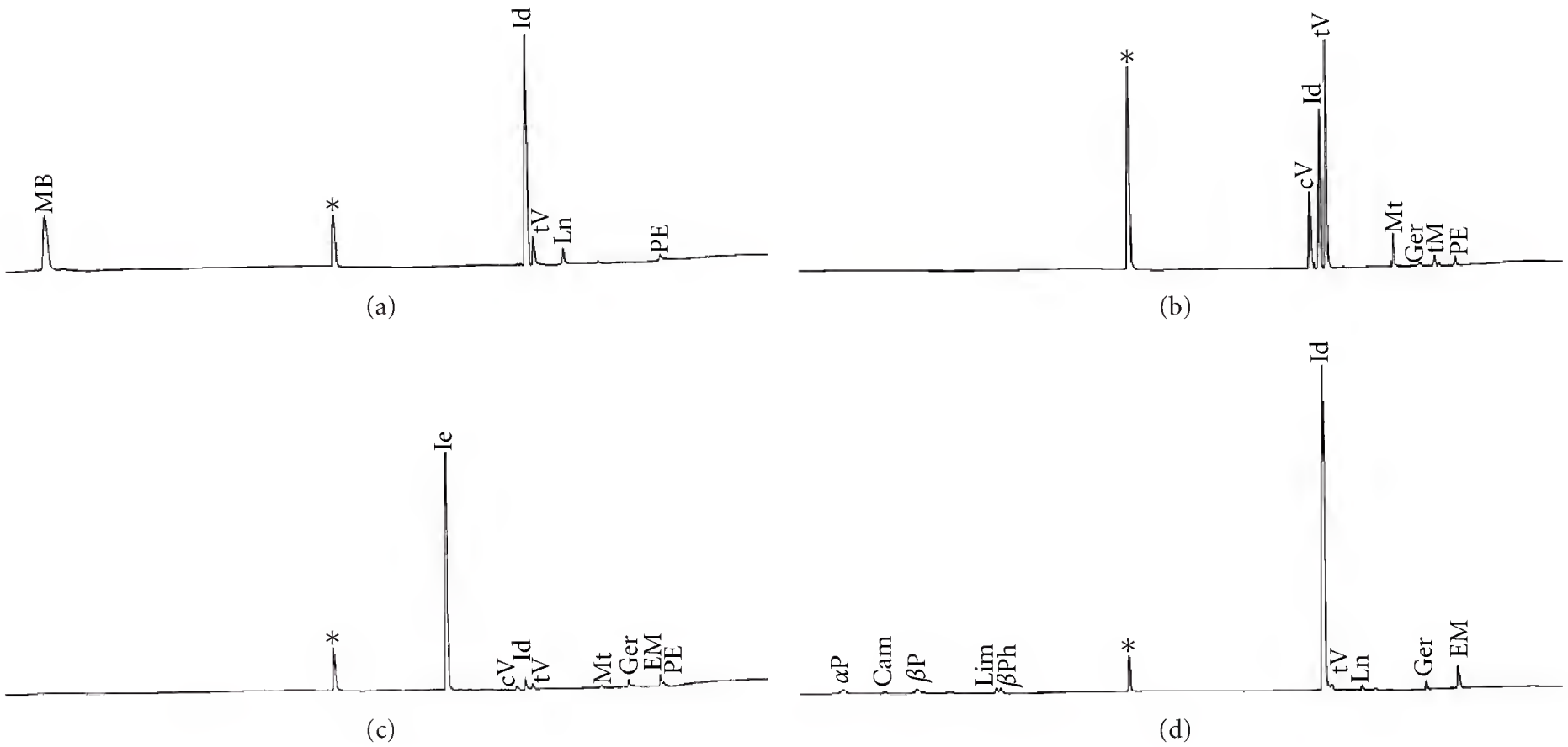

FIGURE 1: GC chromatograms from GC-MS analyses of male hindgut extracts. MB: 2-methyl-3-buten-2-ol; Id: ipsdienol; Ie: ipsenol; cV: *cis*-verbenol; tV: *trans*-verbenol; Ln: lanierone; Mt: myrtenol; Ger: geraniol; tM: *trans*-myrtanol; EM: *E*-myrcenol; PE: 2-phenyl ethanol; ∗: internal quantification standard, heptyl acetate (C_7Ac) amount varies between sample—see the following (for full list of identified compounds and quantities, see Table 4). (a) *I. avulsus* 15 males: C_7Ac is 100 ng. (b) *I. calligraphus* 4 males: C_7Ac is 100 ng. (c) *I. grandicollis* 5 males: C_7Ac is 100 ng. (d) *I. pini* 10 males: C_7Ac is 100 ng.

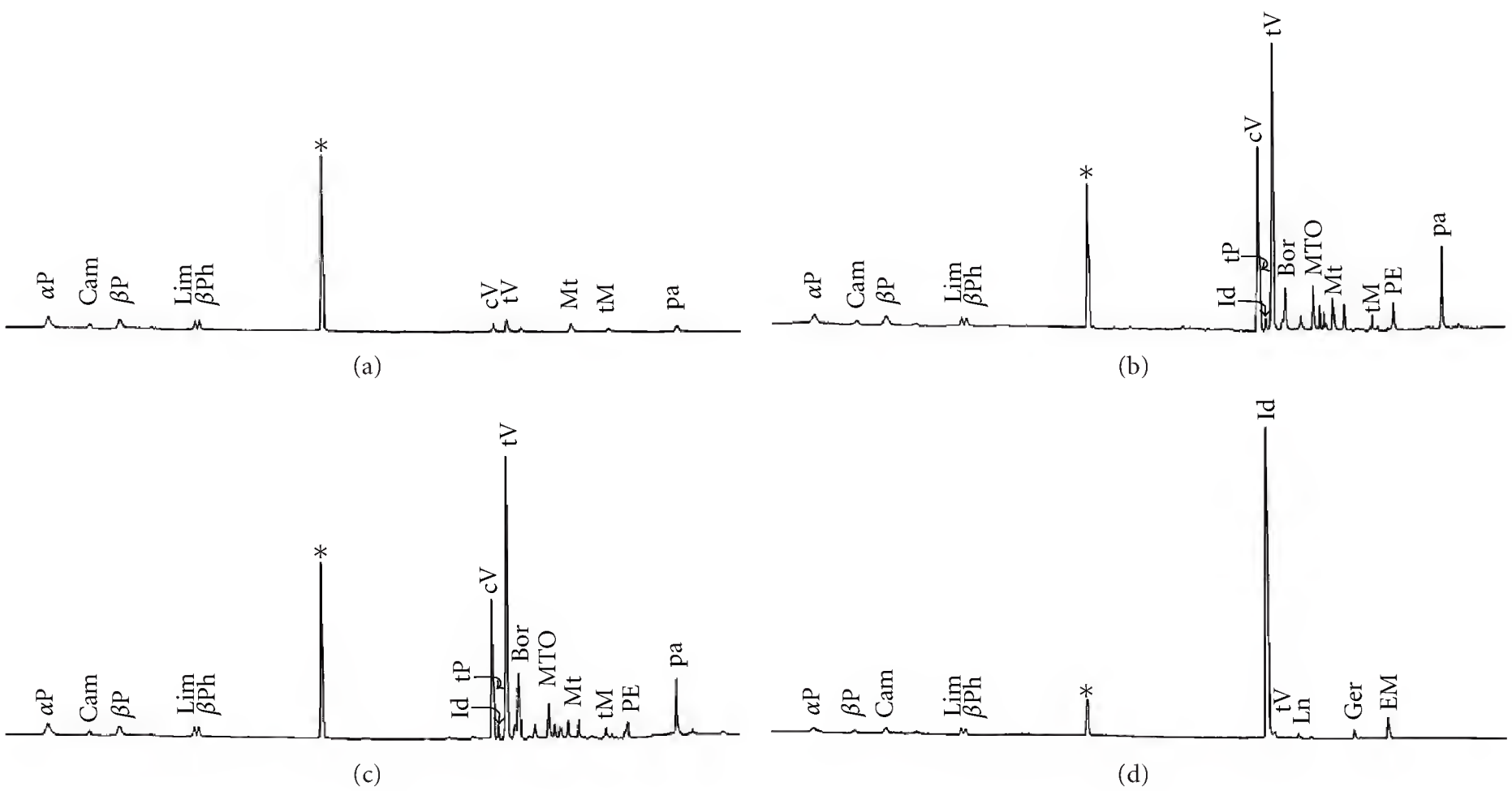

FIGURE 2: GC chromatograms from GC-MS analyses of *I. pini* male hindgut extracts at different overwintering/diapause status. αP: α-pinene; Cam: camphene; βP: β-pinene; Lim: limonene; βPh: β-phellandrene; Id: ipsdienol; cv: *cis*-verbenol; tP: *trans*-pinocarveol; tV: *trans*-verbenol; Bor: borneol; MTO: oxygenated monoterpenes; Mt: myrtenol; Ger: geraniol; tM: *trans*-myrtanol; EM: *E*-myrcenol; Pa: perilla alcohol; Ln: lanierone; PE: 2-phenyl ethanol ∗: internal quantification standard, heptyl acetate (C_7Ac) amount varies between sample—see the following. (a) Late fall; in diapauses, 5 males; C_7Ac is 100 ng. (b) Overwintering, cut out of bolts; 8 males; C_7Ac is 1000 ng. (c) Overwintering, cut out of bolts, on new bolts 48 hrs; 5 males; C_7Ac is 1000 ng. (d) Overwintering, cut out of bolts, on new bolts 96 hrs; 10 males; C_7Ac is 1000 ng.

Table 4: Average amounts of volatiles identified in hindgut extracts: ng/unmated male.

Compound	*I.avulses*	*I. calligraphus*	*I. grandicollis*	*I. pini*	
				48 h	96 h
232-metylbutenol	20	—	—	—	—
Ipsenol	—	—	120	—	—
ipsdienol	20	275	~0.5	8.1	725
E-myrcenol	—	—	6.0	5.6	61
geraniol	(+)	6.5	3.0	—	20
lanierone	2.0	—	—	7.4	23
cis-verbenol	2.5	135	~0.5	87	6.0
trans-verbenol	7.5	400	~1.0	170	10
myrtenol	1.0	90	~0.5	11	—
trans-pinocarveol	—	11	—	27	—
trans-myrtanol	—	30	—	4.5	—
cis-myrtanol	—	2.0	—	2.5	—
perilla alcohol	—	—	—	34	—
2-phenylethanol	1.0	15	3.0	11	—
borneol	—	—	—	47	—

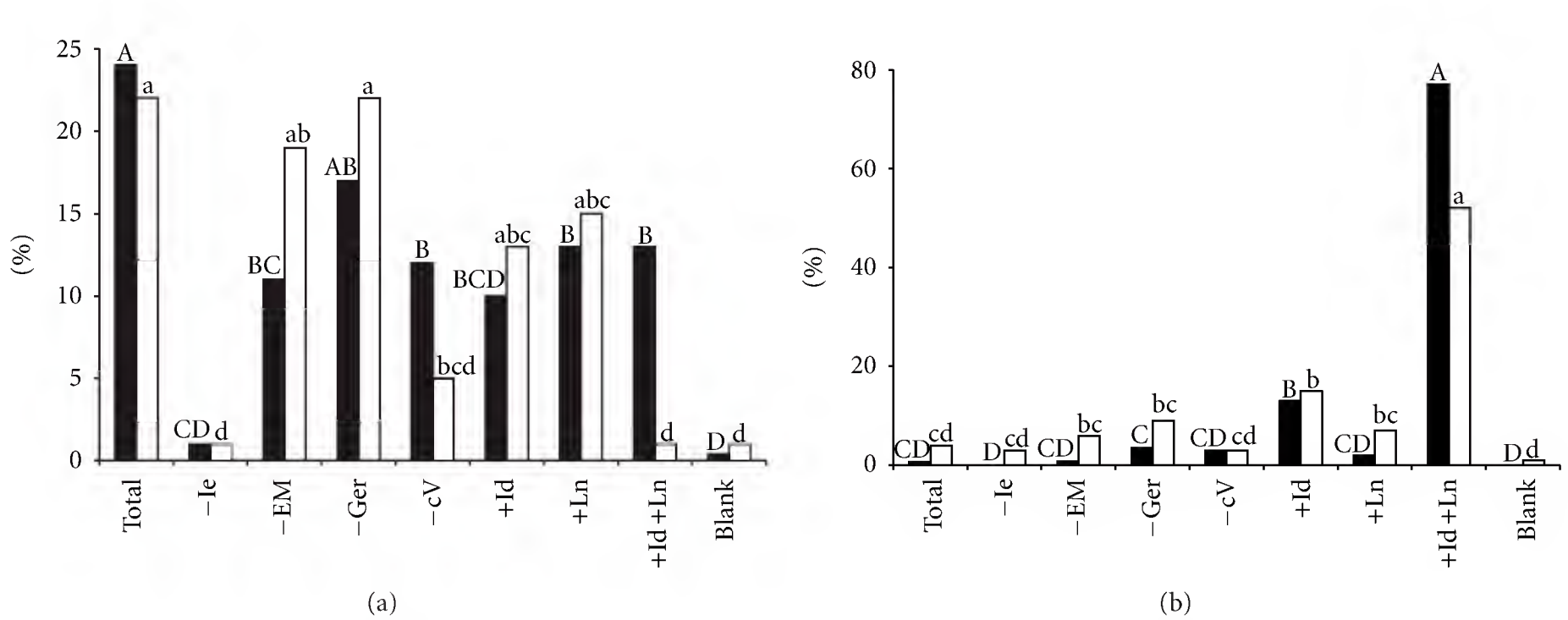

Figure 3: Percentage trap catches in a subtractive and additive field bioassay of *I. grandicollis*-bait: (a) *I. grandicollis* and (b) *I. avulses,* (for bait information, see Table 3). ■ females; □ males. Bars with same letter are not significantly different. $n = 6$; total trap catches: *I. grandicollis* 363 ♀♀, 235 ♂♂; *I. avulsus* 341 ♀♀, 229 ♂♂.

Table 5: Chiral analysis of ipsdienol in three species of *Ips.*

	Ipsdienol	
	(+)-(*S*)	(−)-(*R*)
I. avulsus	85.4%	14.6%
I. pini	61.4%	38.6%
I. calligraphus	21.0%	79.0%

The chemical analysis of hindgut volatiles in unmated males of *I. avulsus* revealed two new pheromone component candidates, 2-methyl-3-buten-2-ol and lanierone. Both of these compounds were included in the field subtractive bioassay, together with ipsdienol and *cis*-verbenol, also identified in the hindgut extracts (Table 4). The results clearly show that ipsdienol is the key pheromone component in this species, as the number of trapped beetles was as low as the blank when this compound was omitted (Figure 4(a)). Lanierone did prove to be a pheromone component of this species, since the trap catch of both sexes were significantly lower without this compound, compared to the full blend. When methylbutenol was excluded, the trap catches were reduced somewhat, but not significantly different from the full blend. The exclusion of *cis*-verbenol had no effect on the trap catch, which suggests that, in *I. avulsus*, this compound is only a detoxification product of the host resin monoterpene (−)-(*S*)-α-pinene, which is unusual for beetles in the genus *Ips* [5, 6]. None of the added compounds, ipsenol and/or *E*-myrcenol, hypothesized to be inhibitory

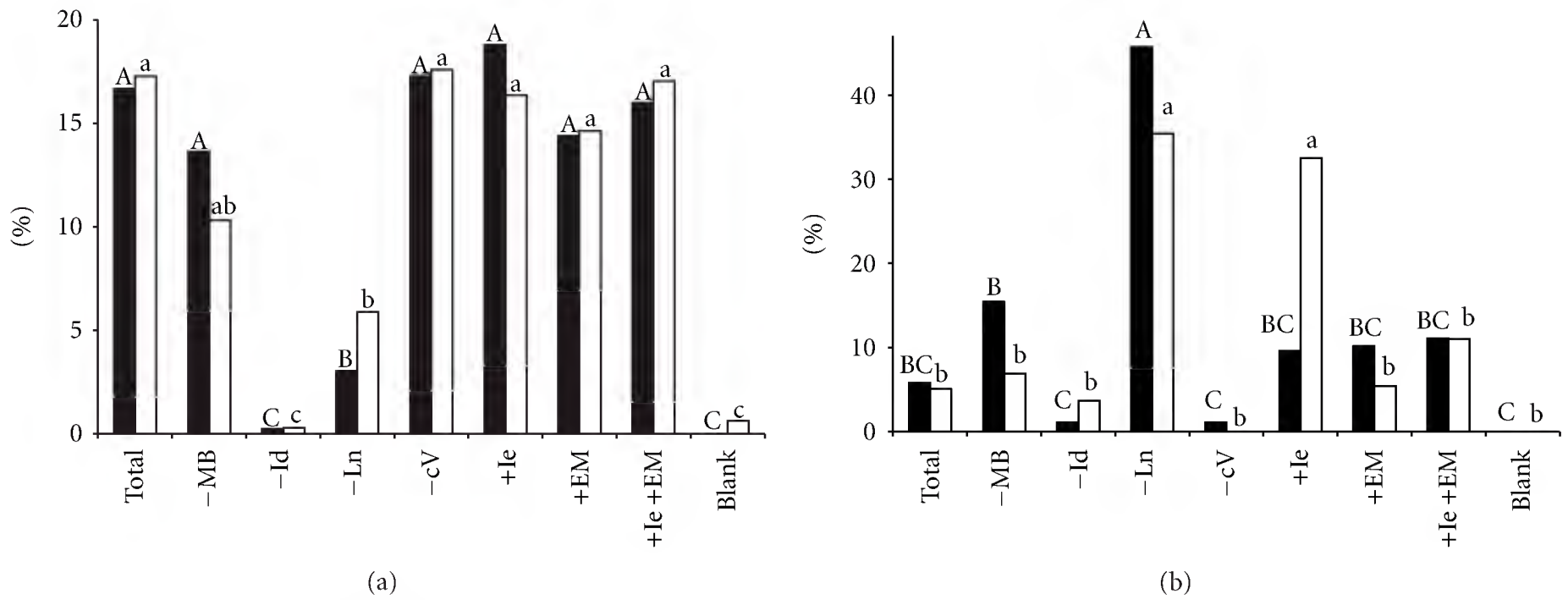

FIGURE 4: Percentage trap catches in a subtractive and additive field bioassay of *I. avulsus*-bait: (a) *I. avulsus* and (b) *I. calligraphus*. (for bait information, see Table 3). ■ females; □ males. Bars with same letter are not significantly different. $n = 7$; total trap catches: *I. avulsus* 1673 ♀♀, 795 ♂♂; *I. calligraphus* 209 ♀♀, 135 ♂♂.

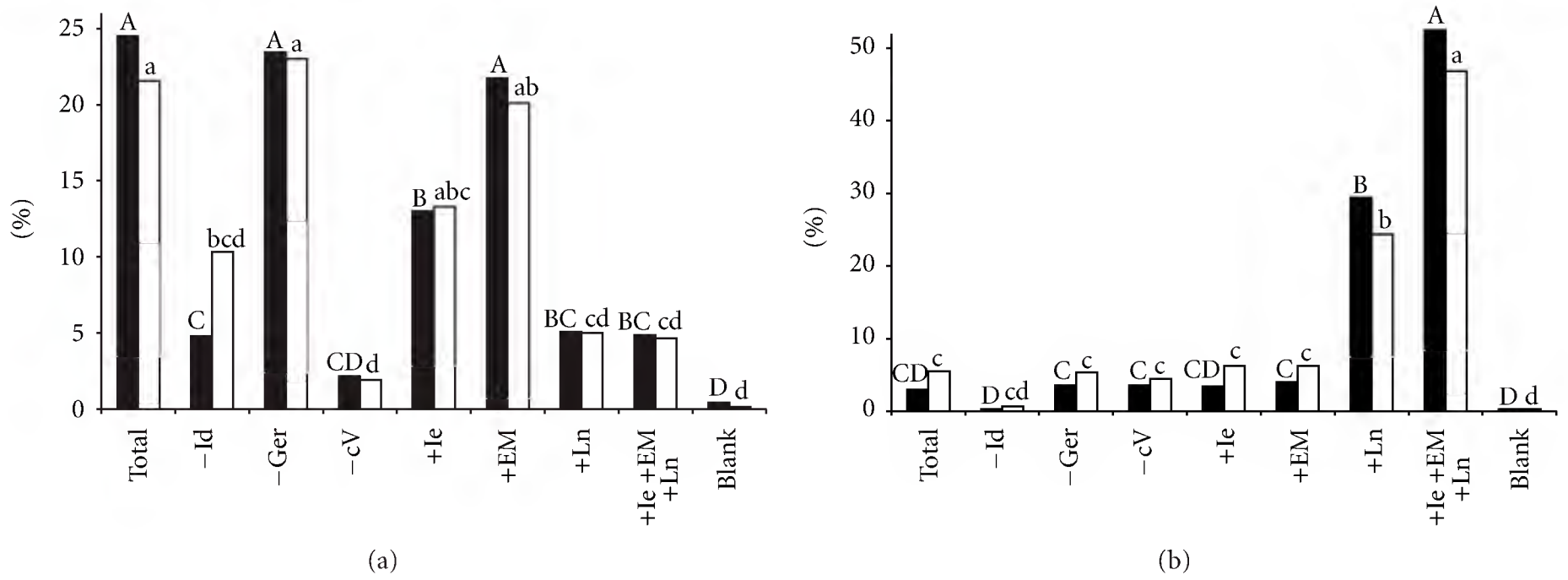

FIGURE 5: Percentage trap catches in a subtractive and additive field bioassay of *I. calligraphus*-bait: (a) *I. calligraphus*, and (b) *I. avulsus*. (for bait information, see Table 3). ■ females; □ males. Bars with same letter are not significantly different. $n = 6$; total trap catches: *I. calligraphus* 523 ♀♀, 378 ♂♂; *I. avulsus* 225 ♀♀, 183♂♂.

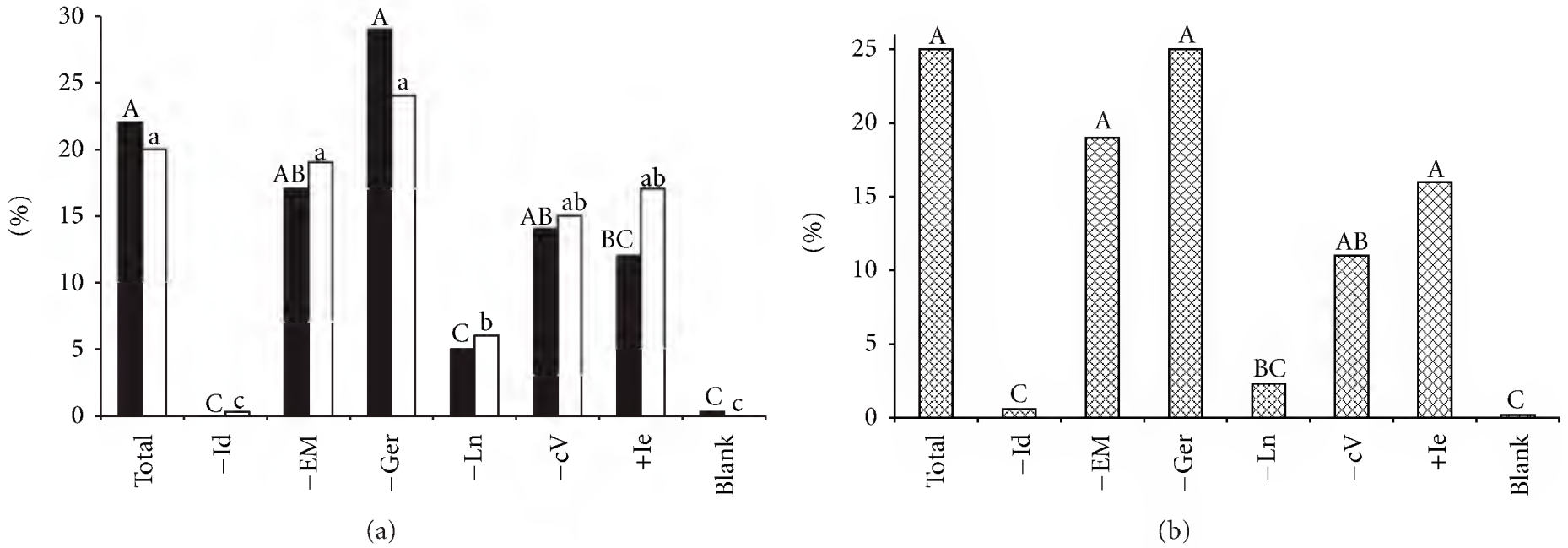

FIGURE 6: Percentage trap-catches in a subtractive and additive field bioassay of *I. pini*-bait: (a) *I. pini* (b) *I. avulsus*. (for bait information, see Table 3). ■ females; □ males. Bars with same letter are not significantly different. $n = 5$; total trap catches: *I.pini* 602 ♀♀, 296 ♂♂; *I. avulsus* 7,000 ♀♀ + ♂♂.

to this species, had any effect on the trap catches on either sex. Most *I. calligraphus* were trapped when lanierone was omitted from the total blend of the *I. avulsus* bait. This indicates that lanierone is an inhibitor to *I. calligraphus* (Figure 4(b)). No other compound in the *I. avulsus* bait had any significant effect on *I. calligraphus*, even though exclusion of methylbutenol gave a higher percentage trap catch than the total bait, however not significantly. Surprisingly, when ipsenol was added to the bait, the number of trapped *I. calligraphus* males increased significantly, compared to the total blend.

The major pheromone candidates in *I. calligraphus* from the hindgut analyses were ipsdienol and *cis*-verbenol. Both these compounds together with geraniol were tested in the subtractive field bioassay, with ipsenol, *E*-myrcenol, and lanierone as possible interspecific inhibitors (Table 3). Both of the suggested pheromone components proved to be necessary for the pheromone blend of this species, while geraniol had no effect on the trap catches (Figure 5(a)). In this species, *cis*-verbenol seems to be the key pheromone component, with very low trap catches for both sexes, which were not significantly different from the blank, when omitted. Exclusion of ipsdienol significantly reduced the trap catches for both sexes, more for females than for males, but not as much as the exclusion of *cis*-verbenol. The addition of *E*-myrcenol did not affect the trap catches, while both ipsenol and lanierone reduced the number of beetles trapped, significantly so by lanierone for both sexes and by ipsenol for females. When all the inhibitory candidates were added, the result was the same as when lanierone was added alone. *Ips avulsus* shares ipsdienol with *I. calligraphus*, but very few *I. avulsus* beetles were trapped on the full bait (Figure 5(b)), and logically the trap catches were even lower when ipsdienol was omitted. The number of trapped *I. avulsus* increased only when lanierone was added to the *I. calligraphus* bait, and it increased even more when all three putative *I. calligraphus* inhibitors, lanierone, ipsenol, and *E*-myrcenol, were added to the full blend.

Ips pini in Georgia is at its southernmost range and is only found at higher elevations. This species also has a diapause, and beetles in the fall are not supposed to be attracted to pheromone. Therefore the subtractive bioassay of this species was undertaken during the early summer. The bait for the subtractive assay was based on the compounds identified in the hindgut extracts, after the beetles had gone through a cold treatment in the laboratory, to mimic a diapause, and were put on new logs for 96 hours (Table 4; Figures 1(d) and 2(d)). Ipsenol was added as a candidate inhibitor. The key pheromone component of this species is ipsdienol, with almost no beetles trapped when this compound was omitted (Figure 6(a)). The exclusion of lanierone from the full blend resulted in low trap catches, significantly less than the full blend, making this compound a pheromone component, synergistically active with ipsdienol. When *cis*-verbenol was omitted, there was a slight but nonsignificant reduction in the trap catch. The subtraction of either *E*-myrcenol or geraniol had no effect on the trap catches, when compared to the total blend. The addition of ipsenol significantly lowered trap catch of females, while the reduction in trapped males was not significant. *Ips avulsus* was also trapped in this assay. However, the number of trapped *I. avulsus* was too high for sexing the beetles (~7000); therefore only results from combined sexes are presented. The trap catch pattern for *I. avulsus* is very similar to that for *I. pini* (Figure 6(b)). When ipsdienol is omitted, almost no beetles were trapped, and the subtraction of lanierone also gave significantly lower trap catches, compared to the total blend. Reduced trap catch due to exclusion of *cis*-verbenol was not significant. Addition of ipsenol did not have any significant effect on the number of beetles trapped.

4. Discussion

The chemical analyses revealed new pheromone component candidates in most species. However, these compounds have earlier been identified and found active in other species of *Ips*. 2-Methyl-3-buten-2-ol in *I. avulsus* was first identified in *I. typographus* by Bakke et al. [22], was found to be a synergist to *cis*-verbenol for that species, and was used in mass trapping programs in Scandinavia and Germany [23–25]. *E*-Myrcenol in *I. grandicollis* and *I. pini* was first identified as a bark beetle pheromone component in *I. duplicatus* [13]. The compound was inactive by itself but increased the trap catches of *I. duplicatus* 25-fold compared to ipsdienol alone. Teale et al. [14] identified lanierone in *I. pini*, in a rigorous aeration, fractionation, and bioassay study. Lanierone was present in *I. avulsus* male hindgut extracts, on the average 2 ng per beetle. When the males of *I. pini* produced their pheromone after cold treatment, there were 10 times more lanierone than found in *I. avulsus*. Besides the new pheromone components, geraniol was identified in all four species of *Ips*, but no pheromonal activity could be found. Therefore, this compound must be regarded as a precursor to the *de novo* produced ipsenol, ipsdienol, and *E*-myrcenol.

The decision regarding which of the identified compounds to include in the subtractive assay of each species was based on three factors. First, each subtractive bioassay should only include compounds that are produced by the species to test. Second, compounds not included in the specific subtractive assay of one species, but included as a pheromone candidate in another species-specific assay, can be added as an inhibitor candidate and should be added individually and in combination. Third, the basis for exclusion of compounds was those regarded as host tree resin detoxification products, that is, oxygenated monoterpenes. This relationship was partly based on the compounds present in *I. pini* during the period after cold treatment and the start of their pheromone production (Table 4 and Figures 2(c) and 2(d)) and in part on experiences with analyses of *Ips* species and other bark beetles [21, 26]. Large quantities of these monoterpene alcohols reflect the amount of resin the beetles have encountered during their excavation of their nuptial chambers. The only exception in this group is *cis*-verbenol, which has been proven as a pheromone component for several species of *Ips* [5, 6], and was therefore included in the bioassays. The last compound excluded was 2-phenyl

ethanol, which has been identified in male hindgut extracts of several genera and species of bark beetles. The activity of this compound is still very unclear, as it has been reported to be an attractant synergist [27], or to have no behavioral impact [26], or even reduce the attraction to pheromone components [28, 29].

4.1. Subtractive Bioassays of Aggregation Pheromones. For *I. grandicollis*, the major result is that ipsenol and *cis*-verbenol are necessary for the pheromone of this species (Figure 3(a)). The subtraction of *E*-myrcenol gave different results in males and females. The subtraction had no significant effect on the trap catches of males but significantly reduced the trap catches of females. Therefore, *E*-myrcenol might act more as a sex pheromone, rather than an aggregation pheromone component.

Two new compounds were identified in *I. avulsus*: 2-methyl-3-buten-2-ol and lanierone. The subtractive bioassay (Figure 4(a)) clearly shows that the aggregation pheromone is made up of ipsdienol and lanierone. When either of these two compounds was excluded, the trap catches dropped significantly for both sexes. However, when the methylbutenol was subtracted, neither of the sexes showed significantly reduced attraction to the bait, even though overall trap catches were reduced. Therefore, we cannot rule out the possibility that methylbutenol might be a behaviorally active compound. 2-Methyl-3-buten-2-ol in *I. typographus* has been found to have a close range attractance [30], apparently promoting landing, as hypothesized by Dickens [31], based on electrophysiological studies. The exclusion of *cis*-verbenol had no effect on the number of beetles trapped, indicating that *cis*-verbenol is not included in the pheromone of *I. avulsus* (Figure 4).

Males of *I. calligraphus* produced very few compounds besides the oxygenated monoterpenes related to the host tree resin. The only two compounds that had an effect on the trap catches were ipsdienol and *cis*-verbenol. Both these compounds are needed for attraction, since subtracting either of them reduces the trap catches dramatically, especially so for *cis*-verbenol (Figure 5(a)).

Ips pini has a wide range in North America, covering the western and northern states of the USA and the southern parts of the Canadian provinces, and following the Appalachian range into the southeastern USA [2]. This species has been reported to be attracted to different pheromone blends in different regions within its range. For many years, this species was thought to have a one compound pheromone, ipsdienol [32, 33]. A decade later, *E*-myrcenol was identified [34] and reported to be behaviorally active in British Columbia [35]. Further investigations on the aggregation pheromone in the New York population of this species resulted in the identification of lanierone another year later [14]. The male beetles analyzed in this study produce, besides ipsdienol, both *E*-myrcenol and lanierone, together with *cis*-verbenol. The subtractive bioassay clearly showed ipsdienol and lanierone to be critical compounds for the aggregation pheromone of *I. pini*, in the present Southeastern population. Lanierone alone does not appear to be attractive alone, as the exclusion of ipsdienol reduced catches to the level of the blank. The exclusion of either *E*-myrcenol or *cis*-verbenol did not have any significant effect on the trap catches, even though somewhat fewer beetles were trapped when *cis*-verbenol was omitted (Figure 6).

4.2. Interspecific Attraction and Inhibition. The results from the pilot subtractive bioassay showed that interspecific inhibition among the four species of *Ips* is very strong, as the most beetles were trapped when one of the compounds was omitted from the total subtractive blend. This was the major reason for performing subtractive bioassays for each species and addition of compounds from sympatric species alone and together to test their effects as inhibitors.

The major difference among the species is the presence of ipsdienol or ipsenol. *Ips grandicollis* is the only species that utilizes ipsenol in its aggregation pheromone, while all the other species produce ipsdienol, as one major aggregation pheromone component. When ipsdienol or lanierone was added individually to the *I. grandicollis* bait, the trap catches were reduced similar to when *cis*-verbenol was omitted (Figure 3(a)). When both ipsdienol and lanierone were added together to the total blend, there were significant reductions in trap catches for both sexes of *I. grandicollis*, and especially so for males. Therefore, male *I. grandicollis* beetles are less likely to land on a host tree from which ipsdienol and/or lanierone are emitted.

In all species-specific bioassays, sympatric beetles were attracted as well as the target bark beetle species, sometimes to the subtractive part, sometimes to the additive part. As the most abundant species, *I. avulsus* was always attracted to the same treatment in the bioassays. In the *I. grandicollis* assay, *I. avulsus* was only trapped when ipsdienol was added (Figure 3(b)), and especially so in combination with lanierone. This clearly indicates that there is no cross-attraction from *I. avulsus* to the pheromone of *I. grandicollis*. In addition, *I. calligraphus* was only attracted in the *I. grandicollis* assay when ipsdienol was added alone.

The three other species of *Ips*, excluding *I. grandicollis*, all have ipsdienol in common. Therefore, more of interspecific attraction will be likely. Accordingly, few beetles of *I. calligraphus* were attracted to the full bait of *I. avulsus*. Only when lanierone was omitted, a significant number of *I. calligraphus* were attracted to the bait (Figure 4(b)). Furthermore, when lanierone were added to the *I. calligraphus* blend, there was a significantly reduced number of *I. calligraphus* attracted to the traps (Figure 5(a)). When ipsenol was added to the *I. calligraphus* bait, the reduction in trapped *I. calligraphus* was lower, but still significant. On the other hand, when *I. avulsus* was exposed to the *I. calligraphus* blend, a low number of beetles were trapped (Figure 5(b)), independent of which compound was subtracted. This indicates that there is no strong attraction to "the total blend", nor is there any inhibitor to *I. avulsus* in the *I. calligraphus* aggregation pheromone blend. When ipsdienol was omitted, the trap catches were even lower. However, when lanierone was added to the *I. calligraphus* bait, significantly more *I. avulsus* were attracted, and, surprisingly, to an even higher level

of significance when all three proposed inhibitors to *I. calligraphus*, ipsenol, *E*-myrcenol, and lanierone, were added to the full blend.

Two species, *I. avulsus* and *I. pini*, were found to have very similar aggregation pheromone blends, and their responses were also very similar. Unfortunately, no *I. pini* was trapped in the *I. avulsus* bioassay, since it was conducted on the coastal plain, outside the range of *I. pini*. However, large numbers of *I. avulsus* were trapped in the bioassay for *I. pini*. In fact, the number of trapped *I. avulsus* outnumbered the *I. pini*, 7000 to 900. The trapping pattern for the *I. avulsus* beetles is almost identical to the pattern for *I. pini* in its bioassay, indicating that these two species behave similarly to the compounds tested in this study. In addition, these two species both belong to the *avulsus* group (group IV) of the genus *Ips*, according to Hopping [2].

Ips avulsus and *I. pini* have overlapping ranges only in the southern Appalachians [36, 37], and they may use spatial separations on the host tree. *Ips avulsus* is usually on the limbs and the top, along with *Pityogenes hopkinsi* Swaine, and *I. pini* is usually on the trunk. Neither of these *Ips* species is attracted to the pheromone of *P. hopkinsi* [38].

4.3. Production of "Inactive Compounds". Why do bark beetles produce compounds not included in their aggregation pheromone? There are different reasons why some compounds identified in male hindgut extracts are not used in their aggregation pheromone. Even though most of the compounds identified in the analyses were monoterpene alcohols, their biosynthetic backgrounds differ. Some are detoxification products of toxic monoterpene hydrocarbons in the host tree resin. Very few detoxification products are used by bark beetles as pheromone components. For *Ips* beetles only *cis*-verbenol of all the detoxification products has been proven to be a component of their aggregation pheromones. The presence of this compound is tightly linked to the amount of (−)-(*S*)-α-pinene in the host resin [39, 40]. The chirality of *cis*-verbenol in *Ips* beetles is reported to be the (−)-(4*S*)-isomer. On the other hand, the (+)-(*R*)-enantiomer of α-pinene is always hydroxylated to (+)-(4*S*)-*trans*-verbenol, with no behavioral effect in *Ips* bark beetles [26]. All other cyclic and bicyclic monoterpene alcohols identified in this study (Table 4) have direct connections to monoterpene hydrocarbons in the host tree resin (Bergquist and Birgersson, unpublished).

On the other hand, the noncyclic monoterpene alcohols identified in this study are not detoxification products of host tree monoterpene hydrocarbons. Instead, geraniol, ipsdienol, ipsenol, and *E*-myrcenol are all produced *de novo* through the mevalonic pathway by the beetles [41–43]. The three compounds earlier found to be active in several *Ips* species are all produced via geraniol as a precursor. While either ipsdienol or ipsenol was found to be active in the species investigated here, no strong activity could be assigned to *E*-myrcenol, other than reduce attraction of female *I. grandicollis* when omitted (Figure 3(a)), even though it was identified in both *I. grandicollis* and *I. pini*. In British Columbia, *I. pini* were found to produce and use *E*-myrcenol as a pheromone component [34, 35]. However, the activity of this compound is still not clear, as trap catches were reduced when it was added to ipsdienol, but attacks increased when the compound was applied to pine logs [35]. It is not known if all populations of *I. pini* produce and are able to perceive *E*-myrcenol. The southeastern *I. pini* produce *E*-myrcenol, but they apparently do not use it as an aggregation pheromone component. This is probably not a by-product from the production of ipsdienol since *I. calligraphus*, which produces large amounts of ipsdienol, does not produce any *E*-myrcenol, while *I. grandicollis*, which produces ipsenol, was found to also produce *E*-myrcenol.

The small amounts of ipsdienol found in *I. grandicollis* are probably a by-product from the production of ipsenol, as ipsdienol is supposedly an intermediate between geraniol and ipsenol in the biosynthetic pathway [44]. Besides the noncyclic monoterpene alcohols, the 2-methyl-3-buten-2-ol, identified in *I. avulsus*, is also produced *de novo* via the mevalonic pathway [45, 46]. This compound was first identified in *I. typographus* by Bakke et al. [22] and is used by this species as a pheromone component [10, 26]. The biosynthetic pathway for lanierone is not yet elucidated.

4.4. Chirality of Compounds. Several bioassayed compounds are chiral, that is, having two enantiomeric isomers. Ipsenol and *cis*-verbenol have been identified as only one enantiomer in *Ips* bark beetles, (−)-(*S*)-ipsenol and (–)-(4*S*)-*cis*-verbenol, and the opposite enantiomers have never been shown to have any effect on the attraction. The chirality of ipsdienol, on the other hand, varies among species and populations within the same species [47, 48]. The chirality of ipsdienol varied between the three species of *Ips* (Table 5), but no species produced enantiomerically pure ipsdienol, nor did any species have racemic ipsdienol, similar to analyses by Kohnle et al. [49] and Seybold et al. [48]. However, all bioassays were done with racemic ipsdienol. There could have been different trap catches if the enantiomeric composition found in each species had been used in the subtractive assays, but at the time the main focus was to identify which compounds each species used in their aggregation pheromones. We do not think that the opposite enantiomer in the racemic ipsdienol had an inhibitory effect on the response, as none of the species had enantiomeric pure ipsdienol, and *I. avulsus*, which was farthest from racemic mixture (Table 5), was trapped in very high numbers on *I. pini* bait (Figure 6). Now that the active pheromone components are identified, the most attractive chiral composition of ipsdienol can be identified.

Ips pini is known to have a wide variation in the chiral composition of ipsdienol between different populations throughout its range [30, 31, 50, 51]. There is a correlation between ipsdienol chirality and attractivity of lanierone over the geographic range, with lower response to lanierone with a higher percentage of (–)-(*R*)-ipsdienol. However, more chiral analyses [50, 51] than bioassays have been done [52]. California populations of *I. pini* do not respond to lanierone [51, 52]. This shift from lanierone as pheromone component

Table 6: Compounds active in field bioassays to four species of *Ips*, P: produced by males; PC: pheromone component; I: inhibitor.

Compound	P	PC	I	P	PC	I	P	PC	I	P	PC	I
		grandicollis			*calligraphus*			*avulsus*			*pini*	
232-metylbutenol							X	?				
ipsenol	X	X				X						♀
ipsdienol			X	X	X		X	X		X	X	
E-myrcenol	X	♀								X		
geraniol	X			X			X			X		
lanierone			X			X	X	X		X	X	
cis-verbenol	X	X		X	X		X			X	?	

Table 7: Baits suggested for monitoring bark beetles in the genus *Ips* in southeastern USA.

I. grandicollis	Ipsenol, *cis*-verbenol
I. calligraphus	Ipsdienol, *cis*-verbenol
I. avulsus and *I. pini*	Ipsdienol, lanierone

is possibly due to a selection pressure by a clerid predator [51].

4.5. Conclusions. Only a few compounds have been identified as aggregation pheromone components in the genus *Ips*. The four species of *Ips* in the southeastern USA investigated in this study are no exception, as they share most of their compounds with other species. The production of compounds and their use as pheromone components and/or interspecific inhibitors for each species are summarized in Table 6. The only species that has a unique compound is *I. grandicollis*, which is the only one of the species studied to use (−)-(*S*)-ipsenol as a pheromone component. Compounds not produced by one species, but by a sympatric species, can act as an interspecific inhibitor. Therefore it is not possible to make a common bait for monitoring populations of the four southern species of *Ips*. In order to avoid inhibitors, key pheromone components will be left out. If all identified attractants are included, then inhibition for some species will occur.

To monitor the four species of *Ips* in the southeastern USA, three different baits will be necessary: one for *I. grandicollis*, one for *I. calligraphus*, and one for both *I. avulsus* and *I. pini*. These baits (Table 7) will not only be highly attractive to the target species but will also keep the other beetles out, with exception for *I. avulsus* from the *I. calligraphus* bait.

Acknowledgments

The authors would like to thank Tim McClure for sorting the trap catches, Bob Larimore, Charles Ford (in memoriam), Fort Benning, and Joe Dinkens, U.S. Forest Service, for providing field sites, Professor Hans Erik Högberg, Mid-Sweden University, for the synthesis of lanierone; and Dr. Dan R. Miller, Dr. Brian T. Sullivan, and Dr. Fredrik Schlyter for reviewing the manuscript. Results from this study were presented at the IUFRO Joint Conference February 6-11, 1994, Maui, Hawaii, and a brief summary was published in the proceedings as G. Birgersson, M. J. Dalusky, and C. W. Berisford in 1995. Interspecific attraction and inhibition among four species of southeastern *Ips* bark beetles; *in* F. P. Hain, S. M. Salom, W. F. Ravlin, T. L. Payne, and K. F. Raffa (eds.), Behavior, population dynamics and control of forest insects, proceedings of the IUFRO Joint Conference, February, 1994, pp 12–18. The authors of this paper do not have any financial relation with any commercial identity mentioned in this paper.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 383757, 8 pages
doi:10.1155/2012/383757

Research Article

Impact of Interference Competition on Exploration and Food Exploitation in the Ant *Lasius niger*

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Received 4 February 2012; Accepted 1 March 2012

Academic Editor: Felipe Andrés León Contrera

Competition acts as a major force in shaping spatially and/or temporally the foraging activity of ant colonies. Interference competition between colonies in particular is widespread in ants where it can prevent the physical access of competitors to a resource, either directly by fighting or indirectly, by segregating the colony foraging areas. Although the consequences of interference competition on ant distribution have been well studied in the literature, the behavioral mechanisms underlying interference competition have been less explored. Little is known on how ants modify their exploration patterns or the choice of a feeding place after experiencing aggressive encounters. In this paper, we show that, at the individual level, the aphid-tending ant *Lasius niger* reacts to the presence of an alien conspecific through direct aggressive behavior and local recruitment in the vicinity of fights. At the colony level, however, no defensive recruitment is triggered and the "risky" area where aggressive encounters occur is not specifically avoided during further exploration or food exploitation. We discuss how between-species differences in sensitivity to interference competition could be related to the spatial and temporal predictability of food resources at stake.

1. Introduction

Competition is generally considered as the major force structuring patterns of distribution and abundance in ant communities [1–4]. Both competition by exploitation and competition by interference can be found in ants. Exploitative competition is defined as the capacity for one species, one group, or one individual to find and exploit rapidly a potentially limited resource, thereby making it unavailable to competitors. Competition by interference on the other hand is defined as the capacity to prevent physical access to a resource, either directly by disturbing or attacking other foragers or indirectly, by delimiting a territory and excluding competitors from foraging sites [5]. Interference competition is particularly widespread in ants. Indeed, many ant species show some forms of territoriality [6, 7], and workers from one colony readily attack intruders from other colonies of the same [3, 8] or of a different species [9, 10].

The level of aggression displayed during interference encounters in ants can be tuned according to a variety of factors, including the species to which the competitor belongs [11, 12], the degree of familiarity with the competitor [7, 13–17], and the number of contestants [18–21] as well as the incurred risks in terms of energy/time loss, injury or even mortality [22]. Moreover, the location at which encounters occur [23, 24], the type [20], and quality [25] of resources at stake determine the intensity of aggressive displays. Encounters with intruders can give rise to immediate and overt attacks, accompanied or not by the emission of alarm pheromone, vibrational stimuli, or specific motor displays [26] whose role is to attract nearby nestmates for assistance in excluding competitors. In some cases, individuals instead of attacking, can retreat and recruit nestmates to the location of the encounter (*Oecophyla* [27]; *Pheidole* [12, 28–30]; *Atta* [31]).

Over longer time scale, competition by interference can modify the exploration pattern of ant workers which avoid the location of aggressive encounters [32]. Likewise, ants can tune their food recruitment behavior according to the risk of injury or mortality associated with a feeding place [33, 34]. As regards the aphid tending ant *Lasius niger*, it is known that previous experience with food can influence their exploration behavior [35] but one does not know whether this behavior, as well as the dynamics of food exploitation, can be affected by previous experience with competitors. In this paper we studied therefore the response of *Lasius niger* workers to the presence of a conspecific intruder on their colony home range. First, we tested whether a defensive recruitment is triggered during heterocolonial encounters, that is, whether the workers contacting an intruder recruit nestmates, either locally, in the vicinity of the confrontation or inside the nest, to assist in subduing the intruder. Second, we investigated whether ant colonies subsequently modify their exploration and food exploitation behavior in order to avoid the locations at which encounters with an alien conspecific took place.

2. Material and Methods

2.1. Species Studied and Rearing Conditions. Experiments were run on three colonies of *Lasius niger* collected in September 2010 on the campus of the University of Brussels (50.5°N et 4.2°E, Belgium). All four *L. niger* colonies collected were queenless and contained between 1,000 and 2,000 workers with brood. Colonies were placed in plastic boxes whose walls were coated with Fluon; they nested in test tubes with a water reservoir plugged with cotton at one end. Ants had also *ad libitum* access to test tubes filled with pure water or 0.6 M sucrose. In addition, they were fed every two days with pieces of mealworm larvae (*Tenebrio molitor*). The temperature in the experimental room was maintained around 22°C, and the room was lighted according to a 12 : 12 L : D regime.

2.2. Experimental Setup. During the experiments, the boxes containing the colonies were connected by a T bridge (width: 2 cm, length of each branch: 10 cm) made of foam cardboard to two foraging areas (squared platforms of 6 cm side). The foraging areas were surrounded by Plexiglas walls (height: 2 cm) coated with Fluon to prevent ants from falling off during the experiments. The bridge and foraging areas were covered with pieces of white paper that could be easily replaced so that ants could not be able to use the chemical marks left in previous trials.

2.3. Experimental Protocol. The experiment was divided into four successive phases (Figure 2). The first phase of the experiment lasted 15 minutes and consisted in the spontaneous exploration of the bridge and foraging platforms by the ants. This phase was followed by a 30-minute confrontation phase in which a worker from an alien colony (always belonging to the same colony) was introduced either on the left or the right platform (the position was alternated between replicates). As for most ant species [36], nestmate recognition in *L. niger* is based on cuticular hydrocarbons [37], and workers generally strongly react when contacting a worker from an alien colony. At the end of the confrontation phase, all ants remaining in the setup were captured with a forceps and were placed back in their nest box. We then removed the pieces of paper covering the bridge, replaced them by new ones, and proceeded with a new 15-minute exploration phase. This way, ants could only rely on their spatial memory of the location where they encountered the worker from an alien colony. The second exploration phase was followed by a 30-minute phase of food exploitation in which a bottle cap containing 1 mL of a 0.6 M sucrose solution was placed in the middle of each foraging platform. All phases of the experiment were recorded with a Panasonic WV-BP250 camcorder placed centrally above the bridge, at the level of the bifurcation leading to the two areas. The colonies were starved for two days before the beginning of each experiment. The pieces of paper covering the bridge were not changed between the different phases of an experiment. Walking workers of *L. niger* mark their colony home range passively, by laying cuticular compounds from footprints during exploration [38]. The bridge could thus be marked during the exploration phase. Such an area marking is known to potentiate the attack of conspecifics from other colonies by resident workers. The pieces of white paper however were changed between experiments so that ants could not be influenced in their choice of a branch by any odor left during previous experiments. Three colonies were tested and seven replicates of the experiment were run for each colony.

2.4. Data Acquisition and Statistical Analysis. We counted the flow of ants travelling towards the foraging areas on each branch of the bridge for each minute of the four phases of the experiment. In addition, we counted the number of ants on each foraging area for all phases of the experiment every 3 minutes.

To study the effect of our experimental procedure on the flow of ants on the bridge over the four phases of the experiment, we used a Generalized Linear Mixed Model (GLMM), [39] with the mean flow of ants per minute over the duration of each experimental phase as response variable and experimental phase as a fixed factor. Variation between colonies in the different phases of the experiment and variation between replicates within colonies in the flow of ants on the bridge were accounted for by considering colony and replicate nested within colony as random effect factors, respectively. We used treatment contrast to compare the flow of ants observed during the first exploration phase to those observed during the three other phases of the experiment. The statistical model was fitted with the penalized quasi-likelihood method using the glmmPQL function of the MASS R package with a gamma distribution error.

In order to test whether ants had a significant preference for one of the two branches of the bridge we used a binomial test on the cumulated flow of ants on each branch (expected probability = 0.5) for each replicate of the experiment. To compare the choice of the ants in the different phases of

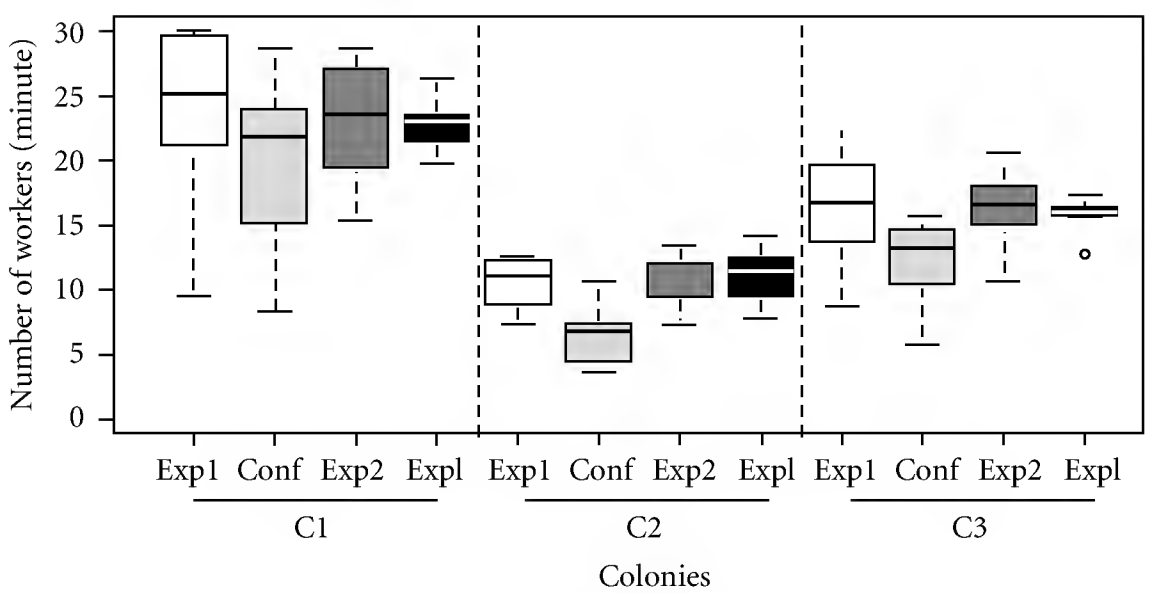

FIGURE 1: Mean flow of workers exiting the nest per minute for the different phases of the experiment and the different colonies (Exp 1 = first exploration phase, Conf = confrontation phase, Exp 2 = second exploration phase, Expl = food exploitation phase) and the different colonies (C1, C2, C3). $N = 7$ replicates per colony.

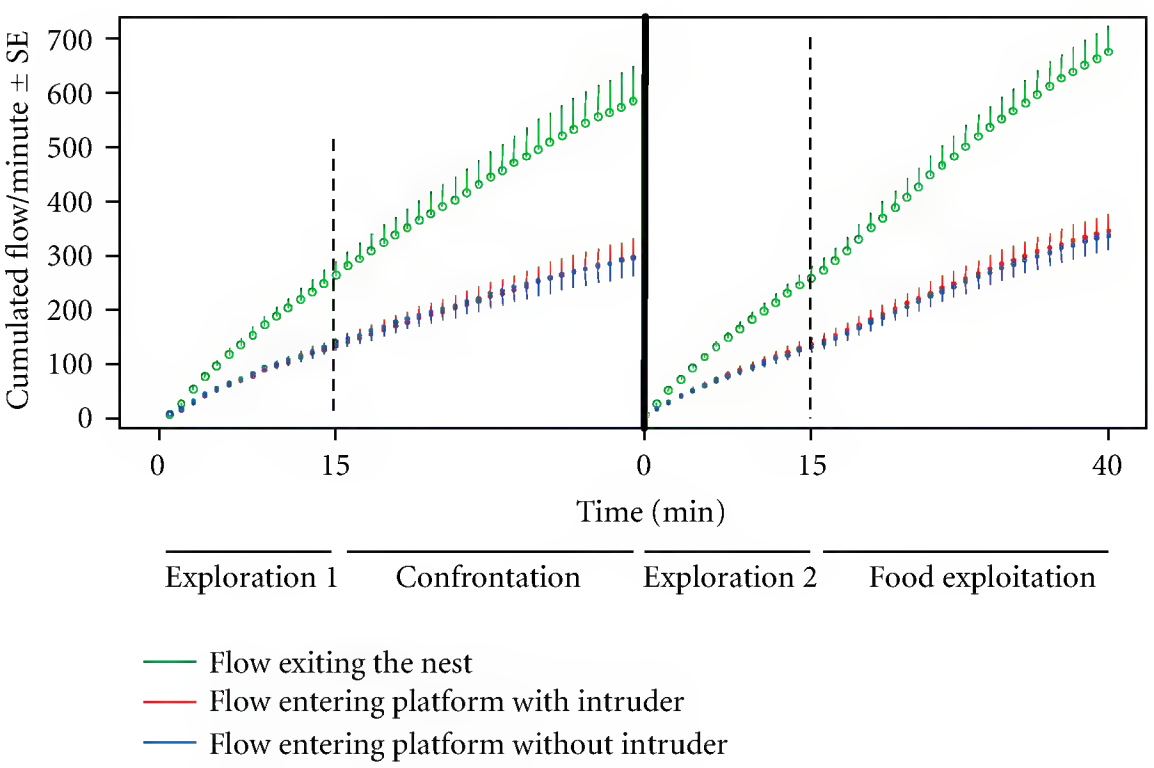

FIGURE 2: Cumulated flow of ants exiting the nest and on each branch of the bridge as a function of time (mean + SE for the flow exiting the nest and the branch where the alien worker was introduced in the confrontation phase, mean − SE for the other branch) $N = 21$ replicates.

the experiment we computed for each phase the number of replicates in which a given percentage of the total flow of ants was observed on the branch of the bridge leading to the platform where the alien ant was introduced in the confrontation phase. A χ^2 test for heterogeneity was then used to compare the distributions. Finally, we examined the sign of the change in the proportion of the total flow of ants exiting the nest towards the risky platform following the exposure to an alien worker. To do so we used a Student's paired t-test to compare, within each replicate of the experiment, the proportion of ants choosing the "risky" branch (leading to the platform where the alien worker was introduced in the confrontation phase) between the first exploration phase and each other phases of the experiment.

All statistical analyses were run with R version 2.13.0 (R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org/).

3. Results

There was an effect of both the phase of the experiment and of the colony on the activity level of the colonies. The mean flow of ants per minute exiting the nest was indeed different among the four different phases of the experiment (Figure 1): the flow observed during the first exploration phase was significantly higher than that observed during the confrontation phase ($t = 3.885$, df $= 60$, $P < 0.001$), but not significantly different to that observed during the second exploration phase ($t = 0.261$, df $= 60$, $P > 0.05$) or the food exploitation phase ($t = 0.377$, df $= 60$, $P > 0.05$). Variation between colonies in the different phases of the experiment was more than four times as important as variation between replicates within colonies. As can be seen in Figure 1, the flow of ants observed in colony 2 was always lower, whatever the phase, than in colony 1 or 3.

In 12 out of 21 replicates, ants did not express a significant preference for one of the branch of the bridge (binomial test, $P > 0.05$) during the first exploration phase. In the nine replicates where they expressed a significant preference for a branch, the right branch of the bridge was always chosen. However, this did not induce a systematic orientation bias for the other phases of the experiment since the branch where the alien worker was introduced was alternatively positioned on the right or left side.

The introduction of an alien worker on one platform during the confrontation phase induced neither an increase nor a decrease in the mean flow of ants per minute towards this "risky" area (Figure 2). In 11 out of 21 replicates, ants expressed a significant preference for one branch of the bridge (binomial test, $P < 0.05$). The branch leading to the platform where the alien worker was introduced was chosen in 6 replicates out of these 11 replicates. Overall, the frequency distribution of choice observed during the confrontation phase did not differ from that observed in the first exploration phase ($\chi^2 = 5.46$, df $= 3$, $P = 0.14$). Within each replicate, however, there was a slight but significant decrease in the proportion of ants choosing the branch leading to the risky platform ($t = -2.718$, $P = 0.013$).

During the second exploration phase that followed the confrontation phase, ants did not attempt to avoid the branch where the alien worker had been introduced. The mean flow of ants per minute on the two branches of the bridge was about the same as in the first exploration phase (Figure 2). Ants chose preferentially one branch of the bridge in six replicates out of 21 (binomial test, $P < 0.005$). Only in 2 replicates out of these 6 replicates ants chose the branch where the alien worker had been introduced. Overall, the frequency distribution of choice observed during the second exploration phase did not differ from that observed in the first exploration phase ($\chi^2 = 1.59$, df $= 3$, $P = 0.66$). Compared to the choice of the ants in the first exploration phase, there was no significant difference in the percentage of ants choosing the branch leading to the risky platform ($t = -1.303$, $P = 0.207$). Therefore, the choice of a branch in the second exploration phase was not influenced by the location where the agonistic encounter with an alien worker occurred.

Finally, during the last food exploitation phase, the mean flow of ants per minute on each foraging platform increased in a similar way on the two branches of the bridge (Figure 2). In 17 replicates out of 21, ants expressed a significant preference for one branch, in eight replicates they preferred the branch where the alien worker was previously introduced, and in nine replicates they preferred the other branch. Collective choices of one branch were more frequently observed during food exploitation than during the other phases of the experiment: this is an expected outcome of the amplifying properties of trail recruitment towards a food source [40]. Overall, the frequency distribution of choice observed during the exploitation phase did not differ from that observed in the first exploration phase ($\chi^2 = 56.07$, df $= 3$, $P = 0.11$). Compared to the choice of the ants in the first exploration phase, there was no significant difference in the percentage of ants choosing the branch leading to the risky platform ($t = 0.683$, $P = 0.502$).

As regards to the occupancy level of foraging areas, it deeply varied with the phase of the experiment (Figure 4). The number of ants on each area increased rapidly at the beginning of the first exploration phase, up to the 10th minute where it began to slightly decrease. During the confrontation phase, while the number of ants remained stable on the "safe" area, it increased steeply on the area where the alien ant was introduced and peaked at the 6th minute. On average, ants were more numerous on the risky platform than on the safe one during the confrontation phase. Actually, when an alien ant was present, a local recruitment was launched: while the intruder was seized by a few resident ants, it was attacked several times by other nestmates. During the second exploration phase, the same level of occupancy as during the first exploration phase was observed for both areas. Finally, the introduction of a food source initially induced a slight increase of the number of foragers which was similar over the two foraging areas and remained stable until the end of the experiment.

The frequency distribution of replicates as a function of the proportion of their foragers on the risky platform (Figure 5) was significantly different between the first exploration phase and the confrontation phase ($\chi^2 = 24.22$, df $= 7$, $P = 0.001$) due to the local defensive recruitment induced by the presence of an intruder. On the other hand, the frequency distribution of area occupancy was not significantly different between the first and the second exploration phase ($\chi^2 = 5.77$, df $= 5$, $P = 0.33$) and between the first exploration phase and the exploitation phase ($\chi^2 = 3.91$, df $= 5$, $P = 0.56$).

4. Discussion

Physical contact or interference with an alien conspecific resulted in a decrease in the flow of *Lasius niger* ants exiting the nest, the latter orienting themselves equally towards the risky or the safer locations (Figure 3). Therefore, workers having encountered a single alien individual on their home range did not recruit additional workers from the nest for assistance. Although *L. niger* workers did not launch a long-range recruitment, they did react at a local scale by increasing their number in the vicinity of agonistic encounters (Figure 4): as soon as an ant entered the risky area, it began trying to subdue the intruder. This temporarily prevented it from returning to the nest to recruit nestmates. The extended staying time of the ants on the risky area may therefore explain the absence of long-range defensive recruitment. One could argue, however, that such a defensive recruitment would be useless since the alien ant was outnumbered by resident ants, right from the beginning of its introduction on the foraging area. It would be interesting to know whether recruitment occurs in *L. niger* when cooperative defense is really useful, that is, when resident ants are outnumbered by intruder ants or when resources that can be monopolized by intruders are at stake.

After the confrontation phase, ants did not avoid the area where the encounter with the intruder took place. Unlike

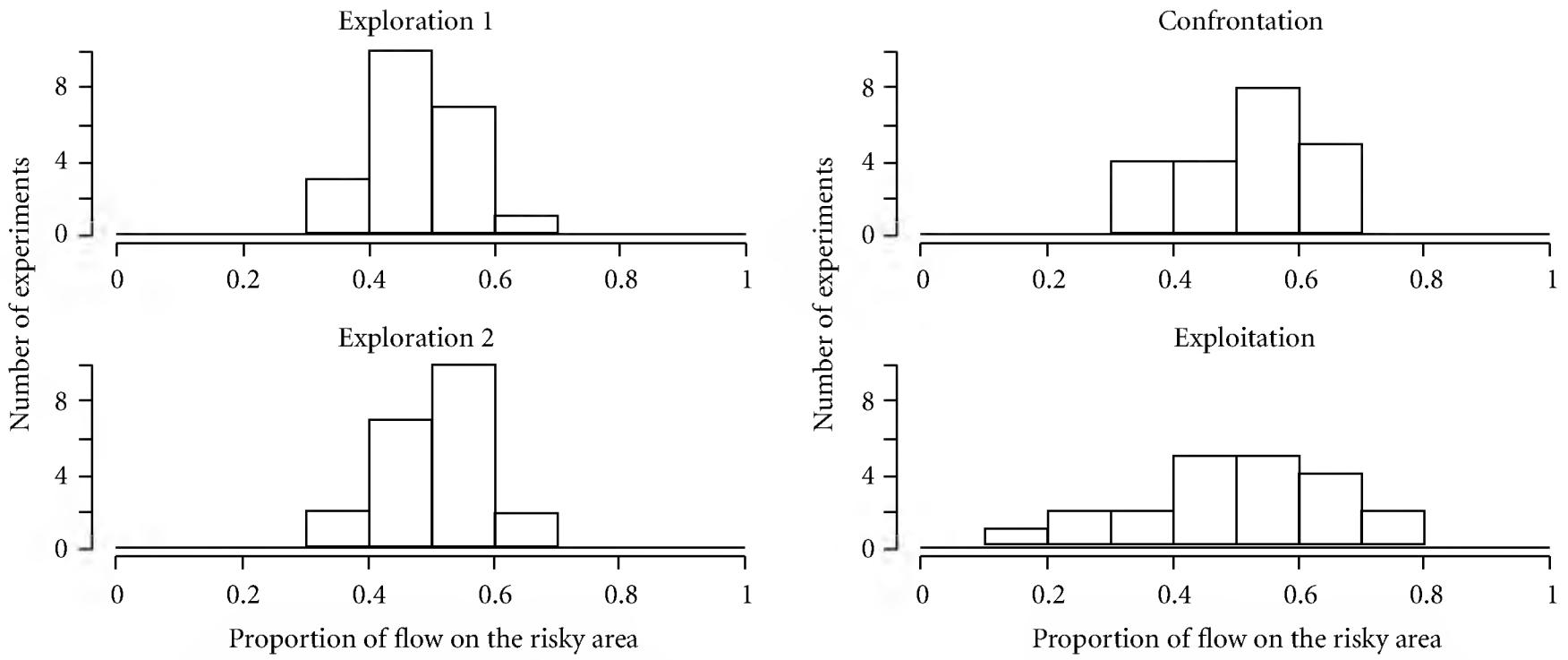

FIGURE 3: Distributions of all replicates as a function of the proportion of ants that were heading towards the area where the alien ant was introduced. The proportion values were calculated over the cumulated flows of ants observed at the end of each phase of the experiment. $N = 21$ replicates.

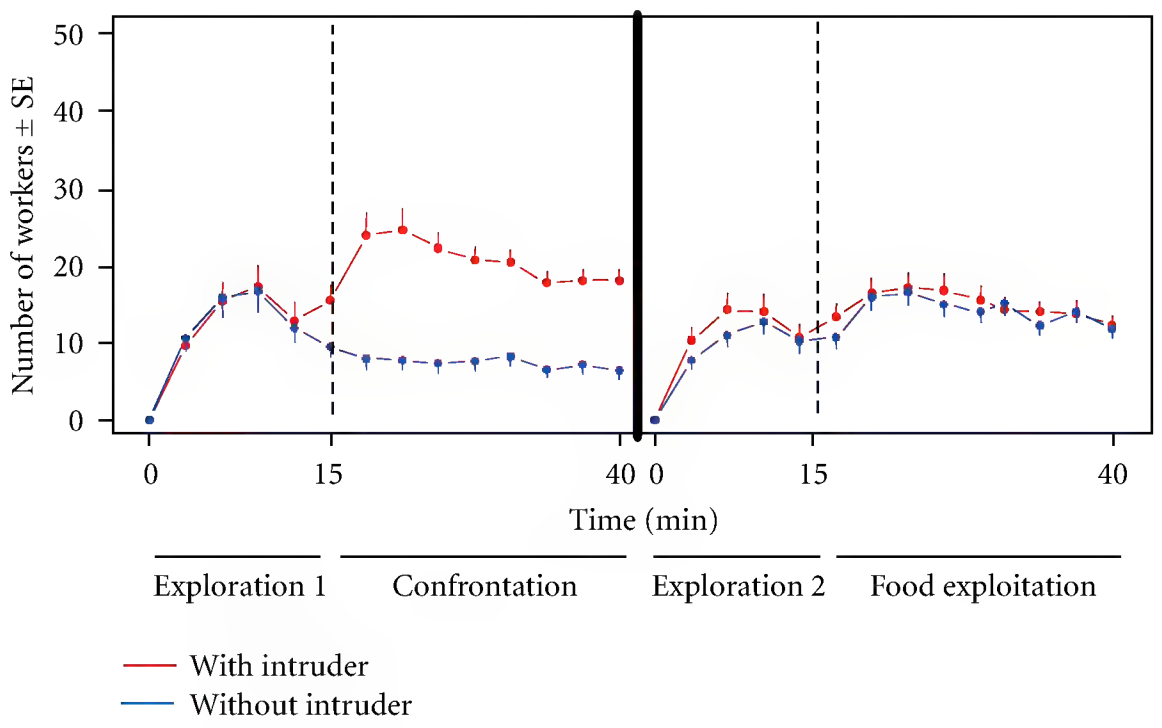

FIGURE 4: Number of ants (mean + SE for the platform where the alien worker was introduced, mean − SE for the other platform) observed every 3 minutes on each platform of the bridge as a function of time. $N = 21$ replicates.

Formica xerophila workers that avoid a location where they have had a negative experience [32], *L. niger* colonies showed the same dynamics and pattern of exploration before and after experiencing agonistic interactions. Likewise, they did not avoid or reduce recruitment intensity towards a food source discovered on a potentially risky location where they had previously experienced aggression.

It would seem logical for ant colonies to avoid potentially dangerous areas. Therefore, one may wonder why in our experiment *L. niger* colonies failed to specifically alter their level of exploration and food exploitation after being exposed to interference competition.

First, one could argue that a single alien worker did not represent a threat high enough or that the exposure time to the threat was not long enough to elicit an avoidance response. However, in the Argentine ant *Linepithema humile*, a single 3 min encounter with a heterocolonial conspecific is enough to produce a long-lasting effect, increasing the propensity to fight in encounters up to a week later [41]. In the same way, in *Lasius pallitarsis*, a short encounter with a single potentially lethal enemy is enough to induce the avoidance of associated food patches even 18–24 h after the encounter occurred [33]. Thus, in our experiment, *L. niger* ants had ample time to perceive interference competition interactions: the intruder was not immediately killed but was physically attacked by several resident workers during the 30 minutes of the confrontation phase.

Second, one could object that ants did not have enough time to develop a spatial memory of the location where the aggressive encounter took place. *L. niger* workers, however, are known to have a well-performing spatial memory [42, 43]. For instance, using a T-bridge similar to our experiment, Grüter et al. [44] showed that after one single visit to a food source, most *L. niger* workers were able to orient to

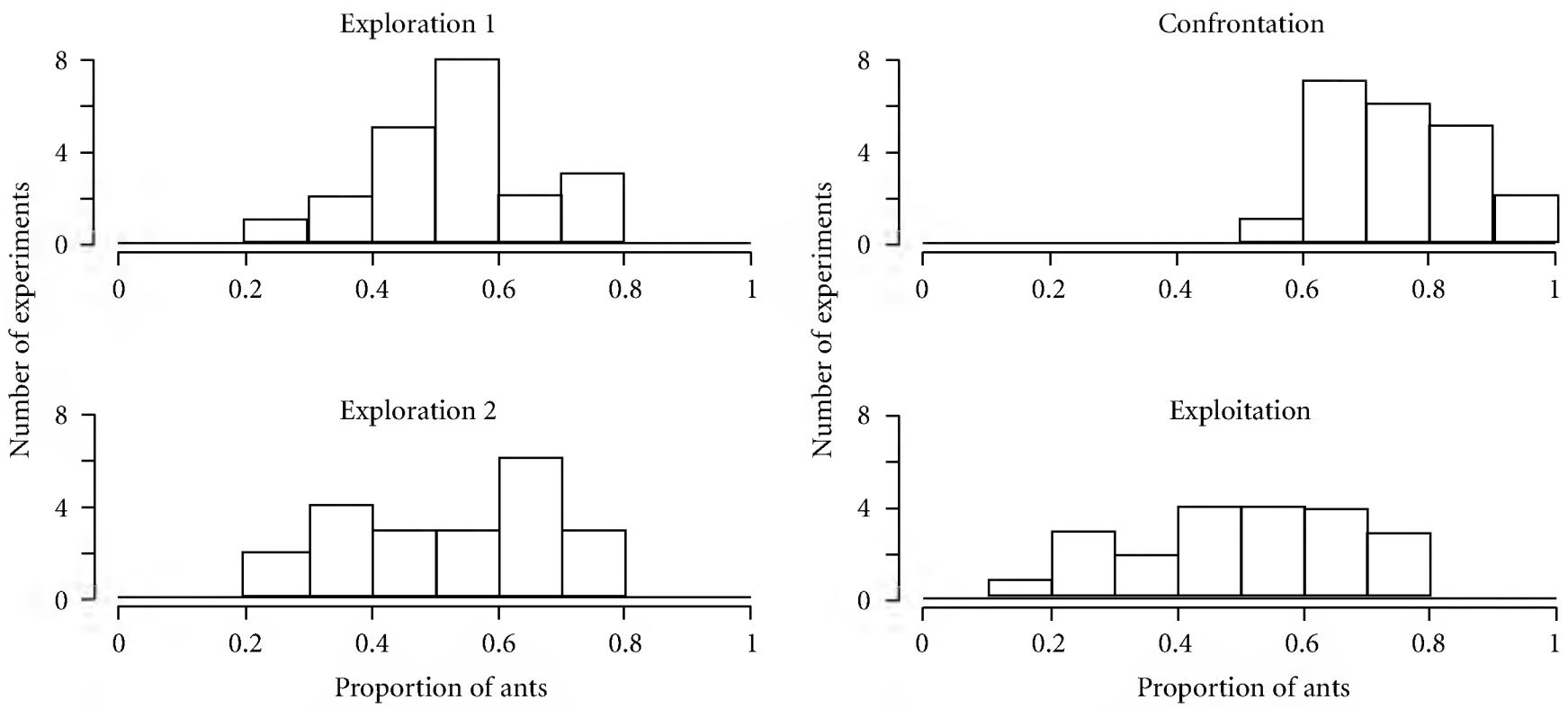

FIGURE 5: Distributions of all replicates as a function of the proportion of workers on the area where the alien ant was introduced (among all foragers present on the two areas). $N = 21$ replicates.

the branch associated with the food source, only on the basis of visual landmarks.

Third, the ability of an ant species to adjust its exploratory, foraging, and defense strategies to match the surrounding competitive risks is likely to be under natural selection pressure and thus strongly correlated to its ecology [24]. Specifically, interference competition interactions may lead to distinct territorial and foraging strategies according to the characteristics of the food resources at stake. For example, temporally and spatially variable food sources such as prey or seed patches are exploited by several ant species. The ephemeral availability of such resources makes the maintenance of absolute territories costly and difficult to achieve. Therefore, a high sensitivity to competition pressure seems well suited for those ants exploiting ephemeral resources allowing them to adjust in a flexible way their exploratory/food exploitation behavior and thereby to reduce the overlap of feeding areas between competing neighbor colonies. In contrast to prey or seed patches, aphid colonies provide stable and renewable resources. Since ants continually require carbohydrates from honeydew to sustain their daily activities, aphid-tending ants such as *L. niger* must maintain access to such resources, even when there is an associated risk of competition. Aphid tending ants thus may prioritize the stabilization of foraged areas by being poorly sensitive to punctual interference competition interactions. Since *L. niger* lives in environments where contacts with competitors are inevitable, defense of aphid resources could be achieved through a local enhancement of agonistic behavior at key locations, such as the vicinity of aphid colonies or the foraging trails. When the competitive pressure becomes higher, the active recruitment of defenders from within the nest will then determine the colony ability to dominate and displace competitors or to abandon the food resource.

Since aggressive behaviours can be costly in terms of energy, time, and physical injuries, any information regarding competitive pressure should be integrated at the colony level to shape the exploratory and foraging strategies of the colony. However, our study reveals that the integration of such information can vary among ant species, *L. niger* being weakly sensitive to previous exposure to a limited interference competition. To fully understand the decision-making process of ant colonies, species-specific responses to agonistic stimuli will have to be investigated in different contexts, such as when the resources at stake are of different quality. Indeed, since in nature food sources vary in their spatiotemporal availability, as well as in their associated risk, colonies may have to make complex decisions: this should involve tradeoffs between the monopolization of rewarding areas under normal competition conditions and the avoidance of dangerous areas through a high spatial flexibility of their home range.

Disclosure

The authors declare that they do not have any direct financial link with any of the commercial identity mentioned in this paper.

Acknowledgments

This work was financed by a PICS exchange program (2008-10) between UMR CNRS 5169 (University Toulouse III) and the Unit of Social Ecology (ULB) as well as by a FRFC Grant 2.4600.09. from the Belgian National Fund for Scientific Research. D. Claire is Senior Research Associate at the Belgian National Fund for Scientific Research.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 978019, 9 pages
doi:10.1155/2012/978019

Research Article

Pollination Requirements and the Foraging Behavior of Potential Pollinators of Cultivated Brazil Nut (*Bertholletia excelsa* Bonpl.) Trees in Central Amazon Rainforest

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Received 5 December 2011; Revised 7 March 2012; Accepted 25 March 2012

Academic Editor: Tugrul Giray

This study was carried out with cultivated Brazil nut trees (*Bertholletia excelsa* Bonpl., Lecythidaceae) in the Central Amazon rainforest, Brazil, aiming to learn about its pollination requirements, to know the floral visitors of Brazil nut flowers, to investigate their foraging behavior and to determine the main floral visitors of this plant species in commercial plantations. Results showed that *B. excelsa* is predominantly allogamous, but capable of setting fruits by geitonogamy. Nineteen bee species, belonging to two families, visited and collected nectar and/or pollen throughout the day, although the number of bees decreases steeply after 1000 HR. Only 16, out of the 19 bee species observed, succeeded entering the flower and potentially acted as pollinators. However, due to the abundance, flower frequency and foraging behavior of floral visitors, it was concluded that only the species *Eulaema mocsaryi* and *Xylocopa frontalis* could be considered relevant potential pollinators.

1. Introduction

Brazil nut (*Bertholletia excelsa* Bonpl., Lecythidaceae) is native from the Amazon forest occurring in the wild from 5°N to 14°S in Venezuela, Colombia, Peru, Bolivia, Suriname, Guyana, and Brazil [1–3]. It is harvested for its nut, which is extracted from inside the large, rounded and hard-to-break fruit collected on the ground after falling from the trees [4]. Most production is for export comprising an important source of food and income to the indigenous people [5].

Brazil nut is believed to be an allogamous species presenting mellitophilous pollination syndrome, thus depending on biotic pollinators to set fruits [6]. However, little is known about its breeding system and pollination requirements. The blooming period occurs from September to December, peaking in November, and flowers are produced profusely in vertical terminal panicles [6, 7]. The flower is large (c.a. 3.9 cm in length × 3.6 cm in width), zygomorphic, with two to three sepals, and six yellowish petals [6, 8]. It bears a curled hood made of congruent staminodes, called ligule, that in association with the petals form a chamber which conceals stamens, stigma, and nectaries [8, 9]. The large size and strength of the hood restricts and selects flower visitors to medium- and large-sized bees strong enough to uncurl it [7, 8]. Anthers begin to dehisce while the flower is still closed, around 0100 HR-0130 HR and over 90% of anthers are shedding pollen by 0300 HR. Pollen viability ranges from 76% to 86.5% and remained viable until 1400 HR [10, 11]. Anthesis takes place between 0430 HR to 0500 HR, and petals fall off after 24 h. When fecundation does not occur, the pistil drops after 48 h [10]. The ovary bears an average of 20 ovules, and only 0.28 to 0.40% of the flowers produced set

fruits [12, 13]. Fruits take an average of 15 months to mature [7, 14].

There are few studies investigating floral visitors of Brazil nut, and usually they are restricted to the genus level. Prance and Mori [15] stated that the main pollinators of species belonging to the Lecythidaceae family are *Bombus* and *Euglossa* bees. Müller et al. [10], dealing with *B. excelsa*, believe that large-sized bees of the genus *Bombus* are the main pollinators of this species, while a study carried out in Bolivia, suggested that euglossine bees are the effective pollinators [13]. However, a study carried out in the state of Acre, Brazil, points out to bees of the genus *Xylocopa* [16]. Only Nelson et al. [9] in a study nearby the city of Manaus, State of Amazonas, and Maués [7], working close to the city of Belém, State of Pará, have identified the bee species visiting Brazil nut flowers to the species level. In both cases, they were all medium-to large-sized bees: *Eulaema seabrai* (Moure, 1960), *Epicharis rustica* (Olivier, 1789), *Ep. umbraculata* (Fabricius, 1804), *Eulaema nigrita* (Lepeletier, 1841), *El. cingulata* (Fabricius, 1804), in Nelson et al. [9] work, and *Xylocopa frontalis* (Olivier, 1789), *X. aurulenta* (Fabricius, 1804), *Ep. rustica* (Olivier, 1789), *Ep. affinis* (Smith, 1874), *Centris similis* (Fabricius, 1804), *El.nigrita, El. cingulata, Bombus brevivillus* (Franklin, 1913), and *B. transversalis* (Olivier, 1789), in Maués [7] report. Recently, Santos and Absy [17] reported *X. frontalis* and *El. mocsaryi* (Friese, 1899), as the most abundant floral visitors of *B. excelsa* flowers in Itacoatiara county, State of Amazonas.

There is a lack of precise information on the breeding system and floral visitors of *B. excelsa*. This work aimed to investigate the pollination requirements, learn about the identity and foraging behavior of visitors to Brazil nut flowers, and discuss their potential as pollinator of this plant species. Such knowledge is remarkably important in developing policies of sustainable use of the forest and conservation of the native bee pollinators. It may also help to explain and to overcome the low productivity observed in commercial plantations of Brazil nut [8–10].

2. Methods

The experiment was carried out in Aruanã farm, situated on the road Manaus-Itacoatiara, km 215, county of Itacoatiara, State of Amazonas, Brazil, at 3° 0′ 30.63″ S and 58° 50′ 1.50″ W. The farm total area comprises 12,000 ha, of which 3,600 are cultivated with 20 varieties of grafted Brazil nut trees. The trees are spaced at 20 × 20 m reaching approximately 1,300,000 trees. It is the largest Brazil nut plantation in the world.

Four trees (three belonging to variety 609 and one to variety Abufari) were chosen at random out of those in blooming. These trees were ca. 0700 HR apart from each other and ranged from 25–30 m in height. Scaffolds were built by the side of each tree, allowing to spot visually 60% of their canopies and access flowers for data collection. Field observations were carried out for 78 days, from October to December 2007, covering the whole flowering period, especially its peak in November.

2.1. Pollination Requirements. Aiming to know the pollination requirements of Brazil nut trees and the role of bees in pollinating this plant species, we applied five pollination treatments to the trees during their blooming.

T1: Open Pollination. We marked 655 buds with satin threads tied to their petiole in the day before flower anthesis. These buds were observed throughout the anthesis and flower lifespan until they have fallen from the trees or being set, until 25 days later. In this treatment, we aimed to know the natural levels of pollination of Brazil nut trees in the area studied.

T2: Restricted Pollination. 326 buds were covered with muslin bags and remained bagged for 25 days. The aim of this treatment was to verify the dependence or nondependence of Brazil nut flowers on biotic pollination.

T3: Hand Cross-Pollination. 150 buds were marked with satin threads and bagged with muslin bags. Next day, after anthesis, flowers were unbagged and manually pollinated with pollen grains from flowers of another Brazil nut tree being deposited directly on the stigma. Donor flowers were collected minutes before we start to perform hand pollination and taken immediately to receptor tree. Then, pollen grains were removed from the anthers of the donor flower using a fine painting brush and transferred promptly to the stigma of the receptor flower. Immediately after hand-pollinated, the flowers were protected with muslin bags for 25 days. This treatment indicates cross-pollination requirements of the brazil nut tree and the existence any pollination deficit by comparison to natural fruit set in the area (open pollination).

T4: Hand Self-Pollination. We marked 98 buds and followed the same procedure described above, except that pollen grains were transferred between anthers and stigma of the same flowers. In this treatment, results show if the Brazil nut tree is self-compatible or not.

T5: Geitonogamy. The same procedure above was repeated here with 78 buds, but pollen grains were transferred from anthers of a flower to the stigma of a different flower from the same tree. We aimed to learn if the Brazil nut tree shows any sort of incompatibility, this kind of crossing and, its dependence on foreign pollen grains.

In this experiment, colors of the satin threads varied according to the treatment, and satin threads were carefully tied to the buds' petiole avoiding damaging the buds, obstruction of the anthesis, and normal development of the flower and fruit set. Also, all hand pollinations were performed between 0600 HR and 0800 HR when, according to Müller et al. [10], fecundity is greatest.

Brazil nut fruits take an average of 14 months to ripe, and other factors besides pollination can interfere with fruit persistence on trees [7, 14]. Thus, in all tests we assessed initial fruit set 25 days after flower manipulation as a measure of pollination effectiveness. This is a reliable measure because unpollinated flowers fall from the trees in the same day they

open, while pollinated ones remain on the trees and show an ovary about 1.5 mm in diameter 25 days later.

2.2. Floral Visitors and Foraging Behavior. Samples of all floral visitors were collected from each tree using entomological nets at every hour from 0500 HR to 1700 HR. Then, insects were killed in a lethal chamber with ethyl acetate, pinned, identified at species level and, sexed, and counted to determine their specific abundance.

During blooming, the foraging behavior of each flower visiting species was recorded considering the following parameters: frequency, abundance, hour of the day and number of visits, time spent per flower, approach and handling of the flower, and entry to the flower. Data were collected using a notepad, a stop watch, a video and photo camera Sony Cyber-shot DSC-H50 9.1 MP, and by means of visual observation of the bees foraging on the flowers, most of them are out of the reach of the observer but in his sight. Recording was initiated when the bee species arrived to the tree and stopped when the insect flew away or went out of the observer's sight, that was limited to only part of the canopy. All data were collected in 25 periods of 30 minutes each, starting at 0500 HR and ending by 1700 HR. This information was later related to temperature, and air relative humidity records obtained every 30 minutes using a digital thermal hygrometer, model Impac TH02, because there are evidences that increases in ambient temperature have a negative impact o the foraging of bees [18, 19].

2.3. Statistical Approach. Data on pollination requirements did not conform to the ANOVA presumptions due to their binomial character (set fruit or nonset fruit) and were analysed using the nonparametric test of Kruskall-Wallis, and means were compared by the nonparametric Dunn's test.

Data regarding the number of flowers visited per tree and time spent per flower were analysed by ANOVA, and means were compared *a posteriori* by Tukey test at 5%. All tests were performed using SPSS 19 Statistics program.

3. Results

3.1. Pollination Requirements. There were significant ($P < 0.05$) differences between treatments for fruit set (Table 1). The hand cross-pollination treatment set the greater number of fruits and differed ($P < 0.001$; KW = 54.295) from all other treatments, while the geitonogamy treatment did not differ ($P < 0.001$, KW = 54.295) to the free pollination treatment. Flowers submitted to the restricted and hand self-pollination treatments set no fruits (Table 1).

3.2. Flower Visitors and Foraging Behavior. Flowers of *B. excelsa* were visited by a wide range of animals, such as Hymenoptera (bees), Lepidoptera (butterflies and moths), and birds (hummingbirds). In Hymenoptera, a great variety of bee species was observed and collected visiting Brazil nut flowers. These bees belonged to two families (Apidae and Megachilidae) in a total of 19 species (Table 2).

Observations on the foraging behavior of floral visitors and potential pollinators showed that bees collect both pollen and nectar from *B. excelsa* flowers. The place from where bees collected nectar from the flowers varied according to the species size. Larger bee species harvested nectar from the ligule base, while smaller species got inside the flower to collect the nectar present at the base of the anthers.

Table 1: Initial fruit set of Brazil nut (*Bertholletia excelsa*) flowers submitted to five pollination treatments: open pollination, bagged with muslin bags, hand cross-pollination, hand self-pollination, and geitonogamy. Itacoatiara, Amazonas, Brazil, 2007.

Treatments	*n*	Fruit set (number)	Fruit set (%)
Free pollination	655	20	3.05[b]
Pollinator exclusion	326	0	0
Hand cross-pollination	159	29	19.33[a]
Hand self-pollination	98	0	0
Geitonogamy	78	3	3.85[b]

*Values followed by the same letters are not significantly different ($P < 0.001$; Kruskal-Wallis ANOVA).

Bees initiated harvesting pollen and nectar at 0515 HR and reached a peak of foraging activity between 0530 HR and 0600 HR. After 1000 HR the number of bees foraging on flowers dropped steeply, coinciding to the temperature increase and relative air humidity drop (Figure 1). However, a small number of bees kept foraging in the afternoon, specially the species *Xylocopa frontalis*. On the contrary of Müller et al. [10] report of bees starting to forage earlier in the dawns following full moon nights, we did not register any difference from the other nights ($n = 2$).

The most abundant floral visitor of Brazil nut was the carpenter bee *Xylocopa frontalis*. This species was the first one to arrive at the flowers (around 0515 HR) to collect nectar and pollen (Figure 2(a)) and was found in great numbers and frequency throughout the whole blooming season of the trees studied. After reaching a flower, *X. frontalis* was used to make a brief inspection of it and, if not rejected, pushed inside the flower using its ligule as a platform to collect nectar from the base of the ligule itself. This bee species was, apparently, the one which carried more pollen on its body, especially on the back of the thorax, head, and in the scopa. A typical behavior observed in *X. frontalis* while foraging was to sit on a flower and groom pollen out of its body towards the scopa and discard with the forelegs the exceeding pollen grains. *Xylocopa frontalis* was among the three bee species that visited most flowers per tree and spent over than 10 seconds per visit (Table 3). Males were observed visiting flowers for nectar, but they also carried great amounts of pollen on their thorax (Figure 2(b)).

Centris denudans (Lepeletier, 1841) was observed visiting flowers (Figure 2(h)) in the canopy of all trees of this study. It was present throughout the blooming season, carrying small amounts of pollen on the back of the thorax, despite the bee large size. This species frequently chased after other individuals of the same species in quick flights over the canopy, possibly to drive the other bee off the food source or to mate with her. It was one of the few species observed foraging in the afternoon, the hottest part of the day,

Table 2: List of families, species, sex and body size of bees, floral visitors, and potential pollinators of Brazil nut (*Bertholletia excelsa*), collected in a commercial cultivation in the county of Itacoatiara, state of Amazonas, Brazil, 2007.

Family	Species	Sex	Body size (mm) ± s.d.
Apidae	*Xylocopa* (*Neoxylocopa*) *frontalis* (Olivier, 1789)	♂♀	34.60 ± 0.10
Apidae	*Epicharis* (*Epicharana*) *flava* (Friese, 1900)	♀	17.40 ± 0.26
Apidae	*Epicharis* (*Epicharana*) *conica* (Smith, 1874)	♂♀	12.30 ± 0.97
Apidae	*Epicharis* (*Epicharis*) *umbraculata* (Fabricius, 1804)	♀	28.70 ± 1.10
Apidae	*Epicharis* (*Parepicharis*) *zonata* (Smith, 1854)	♀	15.20 ± 0.75
Apidae	*Centris* (*Ptilotopus*) *americana* (Klug, 1810)	♀	35.10 ± 0.88
Apidae	*Centris* (*Trachina*) *carrikeri* (Cockerell, 1919)	♂	5.50 ± 1.04
Apidae	*Centris* (*Xanthemisia*) *ferruginea* (Lepeletier, 1841)	♀	7.80 ± 0.45
Apidae	*Centris* (*Ptilotopus*) *denudans* (Lepeletier, 1841)	♂♀	34.20 ± 1.75
Apidae	*Eulaema* (*Eulaema*) *meriana* (Olivier, 1789)	♂♀	33.40 ± 1.20
Apidae	*Eulaema* (*Apeulaema*) *mocsaryi* (Friese, 1899)	♂♀	15.60 ± 0.84
Apidae	*Eulaema* (*Apeulaema*) *cingulata* (Fabricius, 1804)	♀	14.60 ± 0.93
Apidae	*Bombus* (*Fervidobombus*) *transversalis* (Olivier, 1789)	♀	16.40 ± 2.86
Apidae	*Eufrisea purpurata* (Mocsáry, 1896)	♀	10.80 ± 0.89
Apidae	*Eufrisea flaviventris* (Friese, 1899)	♀	15.30 ± 1.33
Apidae	*Apis mellifera scutellata* (Lepeletier, 1836)	♀	4.40 ± 0.19
Apidae	*Frieseomelitta longipes* (Smith, 1854)	♀	1.50 ± 0.24
Apidae	*Melipona* (*Michmelia*) *lateralis* (Erichson, 1848)	♀	4.90 ± 0.32
Megachilidae	*Megachile* sp. 1	♀	4.65 ± 0.76

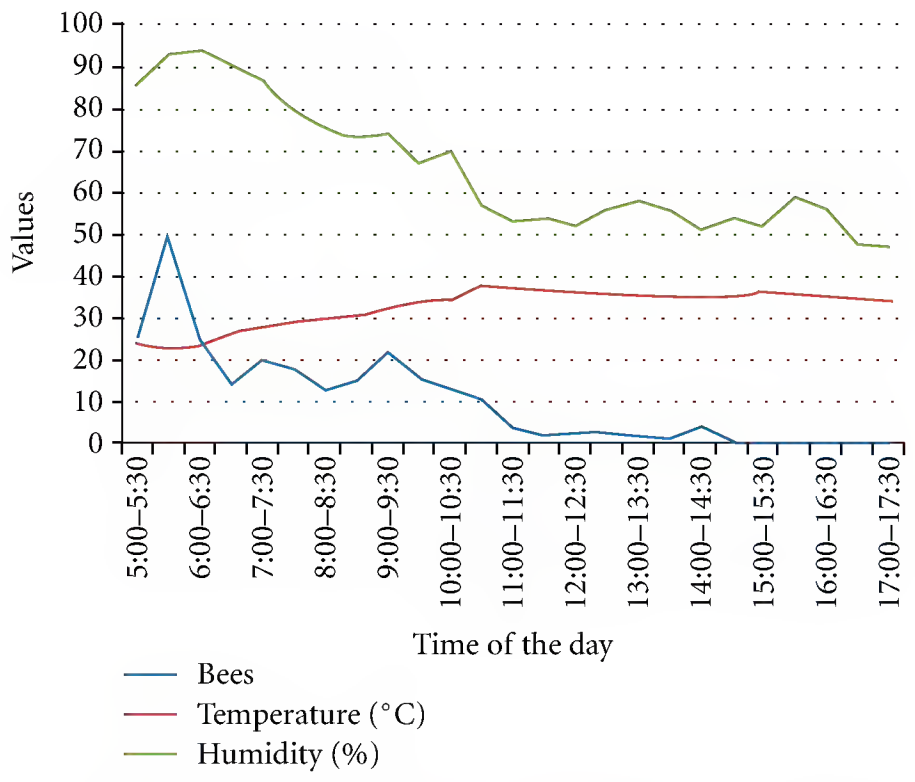

Figure 1: Frequency of floral visitors associated to temperature and relative humidity (at each 30 minutes) in a commercial cultivation of Brazil nut (*Bertholletia excelsa*) in the county of Itacoatiara, state of Amazonas, Brazil, 2007.

although most of its foraging activities were recorded in the morning. This bee species approached the flowers in a different way of *X. frontalis* because it did not inspect and rarely rejected a flower, entering the flower immediately after reaching it, but also harvested nectar from the ligule base. *Centris denudans* ranked second among the species that visited most flowers per tree, usually flowers close to each other, and also spent over than 10 seconds per flower visit (Table 3). Males were observed and recorded visiting *B. excelsa* flowers, and mating events on Brazil nut flowers were also registered.

Eulaema meriana (Olivier, 1789) was also present throughout the blooming season, but only in the morning. Like *X. frontalis*, frequently rejected some flowers but always carried large amounts of pollen in its corbicula. Due to its large glossa, this bee species also collected nectar from the ligule base landing on the ligule itself (Figure 2(k)). *El. meriana* was the bee species that visited most flowers per tree, usually neighboring flowers, spending over than 16 seconds per visit (Table 3). Males of this species were observed harvesting nectar from the Brazil nut flowers.

Centris americana (Klug, 1810) was seen only in some moments of the blooming period and always in small numbers and low frequency to flowers, never exceeding one individual per tree at a given time. This species approached the flower like the other large-sized bees, using the ligule as a platform for landing and collecting nectar from the ligule base (Figure 2(j)). It spent less than 8 seconds per visit (Table 3).

Bombus transversalis was recorded only in the beginning of the blooming season (Figure 2(o)). It was one of the species that spent most time per flower visit, reaching up to 90 seconds inside a flower in some visits. Despite staying long in the flower, *B. transversalis* usually transported small

FIGURE 2: Approach to flowers of Brazil nut (*Bertholletia excelsa*) by distinct bee species in a commercial cultivation in the county of Itacoatiara, state of Amazonas, Brazil, 2007. ((a); (b)) *Xylocopa frontalis* (♀ and ♂, resp.); (c) *Epicharis (Epicharana) flava* (♀); ((d); (e)) *Epicharis (Epicharana) conica* (♀ and ♂, resp.); (f) *Epicharis (Epicharis) umbraculata* (♀); (g) *Epicharis (Parepicharis) zonata* (♀); (h) *Centris (Ptilotopus) denudans* (♀); (i) *Centris ferruginea* (♀); (j) *Centris (Ptilotopus) americana* (♀); (k) *Eulaema (Eulaema) meriana* (♀); ((l); (m)) *Eulaema* (*Apeulaema*) *mocsaryi* (♂ and ♀, resp.); (n) *Eulaema* (*Apeulaema*) *cingulata* (♀); (o) *Bombus* (*Fervidobombus*) *transversalis* (♀); (p) *Eufriesea flaviventris* (♀); (q) *Megachile* sp.1; (r) *Frieseomelitta longipes* robbing pollen from *El.* (A.) *mocsaryi*.

amounts of pollen and visited only a few flowers per tree (Table 3). Due to its medium size, this species entered almost entirely in the flower to collect nectar at the ligule base.

Eulaema mocsaryi was the second most abundant and frequent species over the whole blooming season, mainly in the morning shift (Figure 2(m)) but also observed visiting flowers in the afternoon. It frequently rejected flowers that possibly had been previously visited by other bee. Between two flower visits, while in flight or landing on a leaf, individuals of this species combed pollen from their bodies into the corbicula making large pollen loads. This bee visited less than five flowers per tree moving quickly to other trees

Table 3: Bee relative abundance, mean number (± standard error: SE) of flowers visited per tree by ten bee species and mean time (± standard error: SE), in seconds, spent by twelve bee species per visit to flowers of Brazil nut (*Bertholletia excelsa*) variety 609, under cultivation in the Amazon rainforest (*n*: number of bees recorded per species).

Species	Relative abundance	Number of flower visits per tree			Time spent per flower visit		
	(%)	*n*	X ± S.E.		*n*	X ± S.E.	
Xylocopa frontalis	62.85	136	11.33 ± 0.834	abc	64	11.63 ± 0.754	bcd
Centris denudans	6.84	35	14.71 ± 2.368	bc	64	11.96 ± 0.736	bcd
Centris americana	1.11		—		4	7.73 ± 0.694	cd
Centris ferruginea	0.55	3	3.67 ± 2.667	c	31	9.14 ± 0.854	cd
Eulaema meriana	6.65	17	15.10 ± 2.358	a	57	16.05 ± 1.204	bc
Eulaema mocsaryi	12.20	72	4.36 ± 0.514	abc	48	15.34 ± 1.488	bc
Eulaema mocsaryi (male)		9	8.33 ± 2.677	abc	55	5.68 ± 0.265	d
Epicharis conica	3.88	8	2.75 ± 0.773	c	7	18.39 ± 2.714	bcd
Epicharis flava	0.37	7	4.43 ± 1.288	bc	45	11.86 ± 1.354	bcd
Epicharis zonata	0.92	9	1.67 ± 0.289	c	3	31.38 ± 13.090	a
Eufrisea flaviventris	0.37	6	7.33 ± 3.373	abc	58	5.96 ± 0.983	d
Eufrisea purpurata	0.74		—		4	14.54 ± 5.809	bcd
Bombus tranversalis	3.51	3	6.33 ± 2.963	abc	42	27.61 ± 1.928	a

* Values followed by the same letters are not significantly different ($P < 0.005$; ANOVA).

(Table 3). However, when visiting a flower, *El. mocsaryi* spent over 15 seconds increasing the chance to deposit pollen on the stigma (Table 3).

Epicharis conica (Smith, 1874) was present throughout the blooming season and like *El. mocsaryi* was more frequent in the morning shift, but also present in the afternoon. Due to its small size, this species penetrates the flower almost entirely and unlike the previous species present here, the bee makes a turn inside the flower before leaving it facing out (Figure 2(d)). This bee was the second species that visited less flowers per plant, but took over 18 seconds per visit ($n = 7$) (Table 3). Males also visited flowers and pushed their bodies completely through the petals getting hidden by the ligule while inside the flower (Figure 2(e)). Because of this behavior, their presence was only noticed because the buzzing noisy produced when approaching the flower.

Epicharis flava (Friese, 1900) was present in reduced numbers and only when most trees were in bloom. It carried much pollen on the back of the thorax (Figure 2(c)), outstanding as a potential pollinator of Brazil nut flowers. This bee visited few flowers per tree and spent around 12 seconds per visit (Table 3).

Epicharis zonata (Smith, 1854) is a small bee that like other species of its size gets inside the flower becoming hidden from sight and leaves it facing out carrying small amounts of pollen on its body (Figure 2(g)). This bee was only found in the peak of the blooming season, mainly around 0900 HR. It is a fast-flying bee that moves between trees frequently making difficult to track its path over a single tree canopy. As a consequence, *Ep. zonata* produced the smaller number of flowers visited per tree among all bee species observed in this study, compensated for the longest period of time registered for flower visit (Table 3).

Eufriesea flaviventris (Friese, 1899) is a medium-sized, fast-flying species, and the faster flower visitor observed in this study spending around only six seconds per visit (Table 3), but many times revisiting consecutively the same Brazil nut flower. This was the only species observed to collect exclusively pollen (Figure 2(p)). It also rejected flowers previously visited by other bees and combed the pollen from its thorax to the corbicula while in flight.

Centris ferruginea (Lepeletier, 1841) is a fast-flying, small-sized bee that penetrates the flower almost entirely using the ligule as a platform. It also leaves the flower facing out (Figure 2(i)) and carrying small amounts of pollen on the back of the thorax. Usually was only noticed due to the buzzing sound of its flight over the canopy. This bee species also visited few flowers per tree, favoring cross-pollination (Table 3).

Megachile sp. was the smaller species registered visiting Brazil nut flowers in this study. It penetrated entirely the flower pushing its body among the petals and ligule and also left the flower facing out with small amounts of pollen on its ventral scopa (Figure 2(q)). Because of its size, probably collected nectar from the base of the anthers and stigma, although it s not possible to know for sure because the bee remained hidden inside the flower while sipping nectar. Due to its low frequency and high flight speed, only one visit was registered.

Eulaema cingulata, *Epicharis umbraculata*, *Centris carrikeri* (Cockerell, 1919), and *Eufriesea purpurata* (Mocsáry, 1896) were collected and observed visiting Brazil nut flowers; however, only in rare occasions not allowing even photos to be taken for the two latter species.

Melipona lateralis (Erichson, 1848) was seen only once visiting a flower and captured immediately after leaving the

flower. No further sights were possible until the end of the study.

Apis mellifera scutellata (Lepeletier, 1836) was the only nonnative species recorded in this study, constituting an invading bee in the Amazon ecosystem. It was present in small numbers flying over the canopy, mainly early in the morning. Because of its small size and strength, the bee could not pull the ligule back as a platform as did the larger bee species or push herself among the ligule and petals to get inside the flower as done by other medium and small-sized bees and remained flying over the flowers and landing to collect small amounts of pollen fallen on petals or ligule after the visits by larger bees.

Frieseomelitta longipes (Smith, 1854) was found in the trees all over the morning shift and in greater numbers than *A. mellifera* and, for the same reasons, also did not get assessment of the floral resources inside the flower. However, *F. longipes* showed the behavior of trying to rob pollen from the corbicula of large bees in the moment they were visiting the flowers (Figure 2(r)), sometimes making these bees to give up the flower.

Besides bees, butterflies, hawk moths, and hummingbirds were also seen visiting Brazil nut flowers. Butterflies use to land on the flower and insert their long proboscis to collect nectar at any time of the day. Hawk moths were only present early in the morning, around 0430 HR. They hovered in front of the flowers and introduced their proboscis through the petals to collect nectar. Hummingbirds showed no preference for time of the day, visiting flowers at any time and also hovered in front of the flowers to introduce their beak and drink nectar.

4. Discussion

Results showed that *B. excelsa* did not set any fruit in the restricted and hand self-pollination treatments suggesting that this species cannot bear fruits from pollen grains originated from the same flower and requires biotic pollinators to transfer pollen grains between flowers. According to Moritz and Müller et al. [6, 10] the Brazil nut tree does not set from self-fertilization because this mating system led to less than the 85% ovule fertilization necessary for fruit set. However, the geitonogamy treatment produced over 3% of fruit set indicating that the Brazil nut tree can set fruits when pollen grains are transferred between flowers of the same plant. Also, results of the geitonogamy treatment were similar to the open pollination treatment signifying that the pollination achieved in this commercial plantation could be accounted to geitonogamy. These findings, associated to the much greater fruit set following hand cross pollination indicates that the Brazil nut tree is an allogamous species, in accordance to other authors [6, 8–10].

Our results may explain why the individual plant production is much higher in natural clusters of few Brazil nut trees in the forest than in plantations with hundred of trees. In the natural environment, with much fewer flowers to visit, pollinators may be forced to move between trees and revisit flowers in a much more frequent fashion than when they face a seemly unlimited number of blooming trees.

Although many species visit Brazil nut flowers, only some bee species showed foraging behavior compatible to potential pollinators of this tree. While bees were numerous and concentrated their visits to the morning shift, when flowers presented fresh pollen and were more receptive [10], butterflies, and hummingbirds visited inflorescences at any time of the day, in an inconstant pattern and in low numbers. Hawk moths, however, visited flowers in the dawn, close to the sunrise, but were also scarce. Besides that, the great majority of bee species entered and moved inside the flower increasing the chance to transfer pollen from their bodies to the stigmas, while butterflies, hawk moths, and hummingbirds remained outside the flower and introduced a much smaller portion of their bodies, proboscis for the Lepidoptera and beak to the bird, being less likely to deliver pollen to the stigmas. This behavior, in association to the reduced number of individuals, erratic foraging activities, and time of flower visit, suggests that these groups of floral visitors play little or no role in the pollination of *B. excelsa*. On the contrary, the foraging behavior of most bee species indicates that they can be effective pollinators of Brazil nut flowers, in accordance with the suggestions of Prance and Mori [15], Maués and Oliveira [20], Maués [7], Zuidema [13], and Argolo and Wadt [16].

However, some bee species could not enter the flower or did not show a behavior suggestive of relevant pollinators for Brazil nut. The behavior of *Epicharis conica, Ep. zonata, Megachile* sp., and *Centris ferruginea* approaching the flower facing in and leaving it facing out after turning its body inside the flower can contribute to considerable deposition on the stigma of the flower's self-pollen (self-pollination), showed here to produce no fruits. It may happen because the bee leaving the flower facing out can touch the stigma with the back of its thorax, where the pollen has just been placed by the anthers, resulting, at the best, in a mixture of the pollen bees carried from previously visited flowers with that presently visited being deposited on the stigma. In such a situation, these bee species would not be efficient pollinators of Brazil nut flowers because *B. excelsa* is a predominantly allogamous species [6, 7]. Also, *Apis mellifera, Melipona lateralis*, and *Frieseomelitta longipes* did not manage to enter the flowers and could not pollinate them. Besides that, *F. longipes* sometimes prevented flowers to be visited by legitimate pollinators chasing them away for attempting to rob pollen from their corbicula. Although this specific behavior had not been reported before, Santos and Absy [17] showed that the presence of other insects on the flowers can make some floral visitors, presumably pollinators, to avoid these flowers.

Despite potential pollinators, the rare visits of *Eulaema cingulata*, *Epicharis umbraculata*, *Centris carrikeri*, and *Eufriesea purpurata* to Brazil nut flowers suggest that these species contribute little to the pollination of *B. excelsa*. But their presence in the trees may explain why Zuidema [13] pointed out euglossine bees as likely pollinators of *B. excelsa*, although *E. umbraculata*, *C. carrikeri*, and *E. purpurata* had never before been reported as floral visitors of Brazil nut flowers and *E. cingulata* only once in the study by Maués [7].

Although bees of the genus *Bombus* had been suggested as the main pollinators of *B. excelsa* [10, 12], in the present

study only one *Bombus* species, *B. transversalis*, visited the Brazil nut flowers. Nevertheless, these visits were limited to the onset of the blooming season. Therefore, it is likely that the genus *Bombus* does not consist in a relevant taxon for the pollination of *B. excelsa* in the area studied here. Similarly, *Epicharis flava* and *Centris americana* were not abundant and were selective in relation to the blooming stage and probably are not among the main pollinators of Brazil nut flowers.

Bee species like *Centris denudans*, *Eulaema meriana*, *Eufriesea flaviventris*, *Xylocopa frontalis*, and *Eulaema mocsaryi* were frequent in the area during most of the blooming season and showed body size and flower handling adequate to pollinate *B. excelsa* flowers. However, due to the abundance and foraging behavior in the trees, we identified *Eulaema mocsaryi* and *Xylocopa frontalis* as the most relevant pollinators of cultivated *B. excelsa* in Central Amazonia. It is important to stress that, although these two bee species are the ones that potentially most contribute to Brazil nut pollination under the conditions found in this study, the pollination level achieved in the plantation is the sum of the pollination performed by each bee species that constitute that guild of pollinators, including those species that contributed less to the process [21, 22].

Many of the bee species presented in this study as floral visitors and potential pollinators of Brazil nut are widespread in the Amazon region, and some of them also occur in other Brazilian ecosystems [23–25]. Some of these bee species were also reported in the literature interacting with other plant species and constitute important floral visitors or even pollinators. *Eulaema cingulata* is a pollinator to *Ischnosiphon gracilis* (Rudge) Koern (Marantaceae) and floral visitor of *Solanum stramonifolium* Jacq. (Solanaceae) in a fragment of the Atlantic Forest in NE, Brazil [23, 24]. Vilhena and Augusto [25] identified *Ep. flava* as an important floral visitor of *Malpighia emarginata* in a cerrado area of Central Brazil.

In the Amazon, studies carried out in the same area of this work on the floral biology of *Bellucia grossularioides* (Melastomataceae) and floral visitors of *Bixa orellana* (Bixaceae) reported *El. mocsaryi* and *X. frontalis* as the main visitors of these plant species [26, 27]. However, only recently Santos [17] produced the first report suggesting *E. mocsaryi* as an important floral visitor and potential pollinator of *B. excelsa*. Males of *Eulaema meriana* were observed in the present work visiting flowers of Brazil nut to feed on nectar. According to Williams and Whitten [28], these male bees are pollinators of *Catasetum tricomis* (Orchidaceae), suggesting some level of interdependence among these three species because the orchid provides only essences for the male bees of *Eulaema meriana* attract their conspecific females, but the pollen and nectar necessary for the bee survival and reproduction got to be obtained from other plant species, like the Brazil nut.

These observations support the claim of Kremen et al. [29, 30] that conserving the native vegetation on the surrounding of cultivated areas is essential to keep stable populations of pollinators, such as the bees of the present study, for providing food, nesting, and other resources indispensable for their survival. The lack of effective pollinators in numbers adequate to pollinate the large number of flowers present in commercial plantations of Brazil nut can be a cause for the low tree productivity observed in these areas. Among all species identified as potential pollinators of *B. excelsa* in this study only *X. frontalis* have been reared in rational nest boxes and tentatively managed for pollination of passion fruit (*Passiflora edulis* Sims. f. *flavicarpa* Deg.) in NE Brazil [31]. Investigations on the possibility of rearing and managing *X. frontalis* and other species identified here for pollination of *B. excelsa* are needed.

Acknowledgments

The authors thank three anonymous referees for their substantial contribution to this paper: Dr. Giorgio Venturieri from Embrapa Amazônia Oriental (CPATU) Belém, for his support and relevant information; Agropecuária Aruanã for permission to carry out this study in its farm, logistic support, and use of its facilities; CAPES/Brazil for the M.Sc. scholarship to M.C. Cavalcante and CNPq, Brasília-Brazil for the research fellowship to B.M. Freitas (proc. No. 305062/2007-7).

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 508726, 4 pages
doi:10.1155/2012/508726

Research Article

Data on the Dusty Lacewing Fauna of Northwestern Argentina with Description of a New Species (Neuroptera: Coniopterygidae)

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Received 23 February 2012; Accepted 2 April 2012

Academic Editor: Robert Matthews

Coniopterygidae material collected in two northwestern provinces of Argentina contained six described and one hitherto undescribed species. A list of the determined coniopterygids and the description of a new *Incasemidalis* species is given. Distinctive features of the species belonging to subgenus *Stangesemidalis* also are discussed.

1. Introduction

The number of coniopterygid species hitherto known from Argentina is only 20, which, considering the size of the country and the great variety of habitats and climatic zones, is rather low. Moreover, 8 of them were described recently [1]. On the other hand, almost all of the Coniopterygidae species reported from Argentina (including the recently described one) are present in the northwestern part of the country. On a recent collecting trip to this region, nearly 60 dusty lacewing specimens (mostly males) were collected by M. E. Irwin in two northwestern provinces of Argentina with Malaise traps. The captured coniopterygids belong to six described and to a previously undescribed species. As the country and collector were the same in all cases, these names are not given with the collecting data repeatedly, with the exception of type material of the new species. On the other hand, the main distinguishing features of the three species of *Parasemidalis (Stangesemidalis)* are given, as their recently resolved separation was supported and enhanced by the investigation of the present material. The holotype of the new species is deposited in the entomological collection of the Foundation Miguel Lillo, Tucuman, Argentina. Otherwise the examined material is deposited in the collection of California Academy of Sciences, in the La Plata Museum, Argentina, in the entomological collection of the Foundation Miguel Lillo and in collection of Hungarian Natural History Museum.

2. Distribution Records and Description of a New Species

2.1. *Pampoconis glencrosi* Sziráki, 2009

Examined Material. 1 male, Salta province, 4 km W of Cafayate; 26°04.6′S, 66°00.3′W, 1760 m above sea level (a.s.l.), steep, sandy hillside, 4-5. X. 2009; 1 male, Salta province, 9 km NE of Cafayate; 26°03.0′S, 65°53.8′W, 1586 m a.s.l., dune system, 1–4. X. 2009, l.

Hitherto this species was known only from the holotype from the Calilegua National Park (Jujuy Province, Argentina).

2.2. *Coniopteryx (Coniopteryx) callangana* Enderlein, 1906

Examined Material. 3 males, Salta province, 4 km W of Cafayate; 26°04.6′S, 66°00.3′W, 1760 m a.s.l., steep, sandy hillside, 26-27. IX. 2009; 1 male, Salta province, 8 km S of Cafayate; 26°08.9′S, 66°57.3′W, 1650 m a.s.l., semi stable dunes, 27. IX-1. X. 2009; 1 male, same data, but 1–3. X. 2009.

This species is widely distributed in the Neotropical Region. In Argentina it is known from Buenos Aires and from the northwestern provinces.

2.3. Coniopteryx (Scotoconiopteryx) chilensis Meinander, 1990

Examined Material. 1 male, Salta province, 4 km W of Cafayate; 26°04.6′S, 66°00.3′W, 1760 m a.s.l., steep, sandy hillside, 30. IX-1. X. 2009; 2 males, Salta province, 29 km NE of Cafayate; 25°58.5′S, 65°45.7′W, 1525 m a.s.l., river basin, 26. IX-2. X. 2009.

The known distribution of the species is Chile and Argentina (Salta province).

2.4. Coniopteryx sp.

Examined Material. 1 female, Salta province, 4 km W of Cafayate; 26°04.6′S, 66°00.3′W, 1760 m a.s.l., steep, sandy hillside, 30. IX-1. X. 2009; 1 female, Salta province, 8 km S of Cafayate; 26°08.9′S, 66°57.3′W, 1650 m a.s.l., semi stable dunes, 3–5. X. 2009.

In light of our present lack of knowledge for determination of females for extra-European species of this genus, it is impossible to identify these specimens.

2.5. Parasemidalis (Stangesemidalis) enriquei Sziráki, 2009

Examined Material. 6 males, Salta province, 8 km S of Cafayate; 26°08.9′S, 66°57.3′W, 1650 m a.s.l., semi stable dunes, 27. IX-1. X. 2009; 2 males, same data, but 1–3. X. 2009; 4 males, 5 females, same data, but 3–5. X. 2009; 3 males, 3 females, Salta province, 9 km NE of Cafayate; 26°03.0′S, 65°53.8′W, 1586 m a.s.l., dune system, 1–4. X. 2009.

Taxonomic rank of *Stangesemidalis* González Olazo, 1984 was reduced recently to a subgenus of *Parasemidalis* Enderlein, 1905, simultaneously with description of the given species and recognition of three species of the subgenus [1]. In addition, Figures 12(A)–12(D) of Meinander [2] also suggest with high probability that he referred to *P. (S.) enriquei* and not *P. (S.) subandina*. Investigation of present material supports the validity of all three species belonging to the subgenus *Stangesemidalis*. The most important features of *P. (S.) enriquei* are the very dark (usually black) thoracal sutures, hypandrium with deep, V-shaped incision in caudal view, the truncated paramere, and the ectoproct with several rather strong bristles caudally. (See also the corresponding key in work of Sziráki [3]). Distribution: northwestern provinces of Argentina.

2.6. Parasemidalis (Stangesemidalis) principiae Sziráki et Greve, 2001

Examined material. 1 male, Salta province, 4 km W of Cafayate; 26°04.6′S, 66°00.3′W, 1760 m a.s.l., steep, sandy hillside, 4-5. X. 2009; 1 male, Prov. Salta, 8 km S of Cafayate; 26°08.9′S, 66°57.3′W, 1650 m a.s.l., semi stable dunes, 1–3. X. 2009; 3 males, Salta province, 29 km NE of Cafayate; 25°58.5′S, 65°45.7′W, 1525 m a.s.l., river basin, 2–4. X. 2009. The most important features of *P. (S.) principiae* are the light brown thoracic sutures, hypandrium with deep, V-shaped incision in caudal view, the truncate paramere, and the ectoproct with a single prominent bristle caudally. Distribution: Chile and northwestern provinces of Argentina.

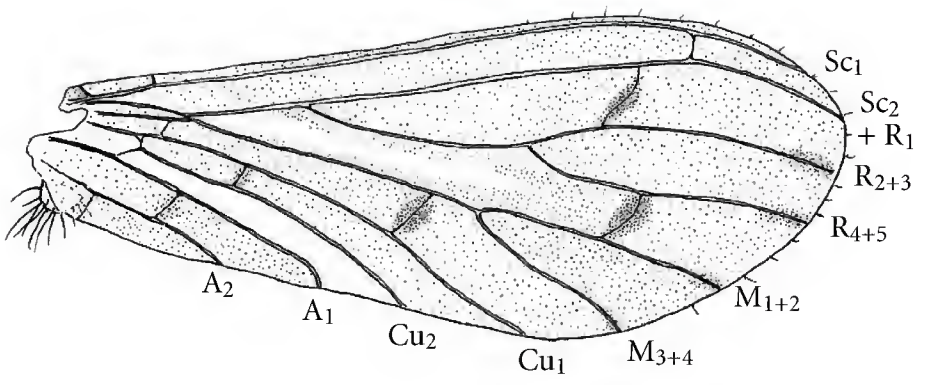

FIGURE 1: *Incasemidalis lineatellus* sp. n., fore wing.

2.7. Parasemidalis (Stangesemidalis) subandina (González Olazo, 1984)

Examined Material. 5 males, Salta province, 8 km S of Cafayate; 26°08.9′S, 66°57.3′W, 1650 m a.s.l., semi stable dunes, 27. IX-1. X. 2009; 5 males, same data, but 1–3. X. 2009.

The most important features of *P. (S.) subandina* are the slightly hooked and in lateral view gradually widened paramere, hypandrium, with wide, U-shaped caudal part in caudal view, and the ectoproct with a few rather strong bristles caudally. (The mentioned shape of the paramere may be recognized in the otherwise somewhat schematic illustration of the original description of the species [4]). Distribution: northwestern provinces of Argentina.

2.8. Parasemidalis (Stangesemidalis) sp.

Examined Material. 1 female Salta province, 8 km S of Cafayate; 26°08.9′S, 66°57.3′W, 1650 m a.s.l., semi stable dunes, 27. IX-1. X. 2009.

The specimen may be either *P. (S.) enriquei* or *P. (S.) subandina.*

2.9. Incasemidalis lineatellus sp. n. (Incasemidalis sp. 1 [3])

Examined Material: Holotype. male, Argentina, Tucuman Province, 22 km SE of Amaicha de Valle, 26°41.5′S, 65°48.4′W, 2900 m a.s.l., steep, dry ravine, 25. IX-1. X. 2009, Malaise trap, leg.: M.E. Irwin; deposited in the entomological collection of the Foundation Miguel Lillo, Tucuman. Paratypes: 5 males, same data as holotype; three of the paratypes are deposited in the collection of California Academy of Sciences, while two others are in Hungarian Natural History Museum, Budapest.

Diagnosis. Membrane of wings moderately spotted. Posterior part of fused stylus + gonarcus plate-like, hypandrium with nearly rectangular caudal projection, main structure of penis in ventral view rectangular.

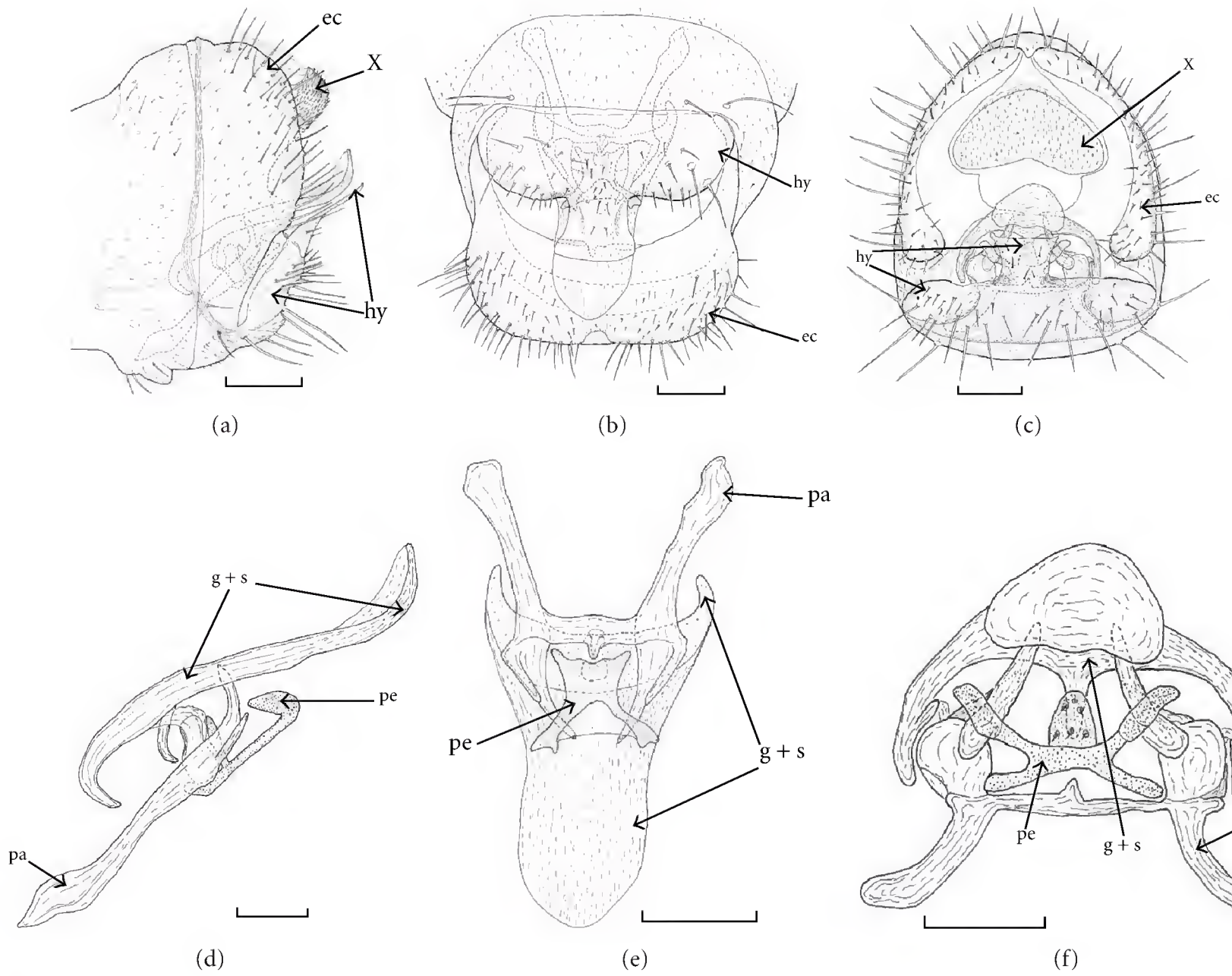

FIGURE 2: *Incasemidalis lineatellus* sp. n., male genitalia. Terminalia in lateral view (a), terminalia in ventral view (b), terminalia in caudal view (c), internal genitalia in lateral view (d), internal genitalia in ventral view (e), internal genitalia in caudal view (f). Abbreviations: ec = ectoproct, g + s = fused gonarcus + stylus, hy = hypandrium, pa = paramere, X = tenth sternite. Scale = 0.06 mm.

Description. Relatively large coniopterygid; body length 3.6–4.0 mm. Frons light brown, vertex medium brown posterior part of head capsule dark brown. Palpi medium brown. Antennae 1.9–2.1 mm, dark brown, 36–41 segmented. Scape about as long as broad, pedicel 1.4 times as long as broad, median flagellar segments 2.7 times as long as broad. Large part of thorax light ochreous, but sutures, large shoulder spots and legs dark brown. Length of fore wing 4.1–4.6 mm, of hind wing 3.5–4.2 mm. Large part of membrane of both wings light brown, but fore wing somewhat darker than hind wing and almost hyaline stripes between Rs-stem of M-Cu_2. Additional dark brown stripes at distal ends of veins R_{2-3}, R_{4+5} and M_{1+2}; distinct narrow, oval spots (which seem to be short dark stripes at low magnification) at cross veins R_1–R_{2+3}, R_{4+5}-M_{1+2} and stem of M-Cu_1 and less marked stripes at the cross veins Cu_1-Cu_2 and An_1-An_2 (Figure 1), as well as at some cross veins of hind wings. (The rather indistinct pattern of hind wing has no taxonomic importance). Wing venation typical for genus, however, on left fore wing of one paratype exist two aberrations, namely, a short longitudinal vein arises from middle of cross vein R_{4+5}-M_{1+2}, while M_{1+2} vein forked for a very short distance just before its end. (Otherwise, the wing venation is of no value in distinguishing among the known species of this genus).

Male genitalia (Figure 2) well sclerotized. Ectoproct short, without any caudal projection. Tenth sternite prominent, with dense short hairs. Hypandrium bears a thin, plate-like, nearly rectangular projection caudally. Dorsal sclerite of internal genitalia, which is regarded by Meinander [5] as the gonarcus, rather may be interpreted as fused gonarcus + styli. Posterior part of this sclerite is plate like, and curved upwards, while its anterior part bears a median and two lateral projections curved ventrally. The lateral projections are connected membraneously to the parameres. Parameres caudally forked, pointed, and ventrally connected by medially knobbed bridge, which probably developed from stylus part of stylus + gonarcus sclerite complex. (It is worth mention that the same bridge ventrally of paramere in some other coniopterygids is generally regarded as a part of the fused styli).

Penis is a nearly quadrate plate, with larger posterior and smaller anterior pair of lateral projections, and dorsally directed hairy median lobe.

Incasemidalis lineatellus sp. n. resembles *I. chilensis* Meinander, 1990 because of the dark spots at some cross veins, the similarity of the stylus + gonarcus sclerite, the presence of the hairy dorsal process of the penis, and because of the presence of a caudal projection of the hypandrium.

The most important diagnostic features of the new species are

(i) presence of dark stripes at the endings of some longitudinal veins and absence between them;

(ii) absence of a caudal knob on the ectoproct;

(iii) presence of a prominent, well-sclerotized tenth sternite;

(iv) the large, plate-like projection of the hypandrium;

(v) presence of a ventral bridge between the parameres;

(vi) the nearly quadrate structure of the penis, with anterior and posterior lateral projections.

Acknowledgments

The authors wish to thank Michael E. Irwin for providing this material and to the Evert I. Schlinger Foundation for the financial support of the collecting.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 591616, 11 pages
doi:10.1155/2012/591616

Research Article

Chemical Constituents and Combined Larvicidal Effects of Selected Essential Oils against *Anopheles cracens* (Diptera: Culicidae)

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Received 27 March 2012; Accepted 5 April 2012

Academic Editor: Kabkaew Sukontason

A preliminary study on larvicidal activity against laboratory-colonized *Anopheles cracens* mosquitos revealed that five of ten plant oils at concentration of 100 ppm showed 95–100% larval mortality. The essential oils of five plants, including *Piper sarmentosum, Foeniculum vulgare, Curcuma longa, Myristica fragrans*, and *Zanthoxylum piperitum,* were then selected for chemical analysis, dose-response larvicidal experiments, and combination-based bioassays. Chemical compositions analyzed by gas chromatography coupled to mass spectrometry demonstrated that the main component in the oil derived from *P. sarmentosum, F. vulgare, C. longa, M. fragrans, and Z. piperitum* was croweacin (71.01%), anethole (63.00%), ar-turmerone (30.19%), safrole (46.60%), and 1,8-cineole (21.27%), respectively. For larvicidal bioassay, all five essential oils exerted promising efficacy in a dose-dependent manner and different performances on *A. cracens* after 24 hours of exposure. The strongest larvicidal potential was established from *P. sarmentosum*, followed by *F. vulgare, C. longa, M. fragrans*, and *Z. piperitum*, with LC_{50} values of 16.03, 32.77, 33.61, 40.00, and 63.17 ppm, respectively. Binary mixtures between *P. sarmentosum*, the most effective oil, and the others at the highest ratio were proved to be highly efficacious with a cotoxicity coefficient value greater than 100, indicating synergistic activity. Results of mixed formulations of different essential oils generating synergistic effects may prove helpful in developing effective, economical, and ecofriendly larvicides, as favorable alternatives for mosquito management.

1. Introduction

Presently, the risk of contracting arthropod-borne diseases has increased due to the climate change and intensifying globalization [1]. Malaria, a life-threatening disease transmitted by mosquitoes, is continuing to be a major public health problem causing death and illness in children and adults around the world, especially in tropical countries. About 3.3 billion people—half of the world's population—are at risk of malaria. Every year, this leads to about 250 million malaria cases and nearly one million deaths [2]. Malaria control requires an integrated approach, including prompt treatment with effective antimalarials and prevention, primarily based on vector control. However, an inappropriate use of antimalarial drugs in the past century contributed to the increasing and widespread drug-resistant malarial parasites in the endemic areas, leading to rising rates of sickness and death. Therefore, mosquito management has played an essential role in the substantial reduction of malaria. The control of mosquito at the larval stage is necessary and efficient in the integrated approach to mosquito management. Mosquito adulticides, although effective, are often

applied only as a temporary solution to disease outbreaks for transiently minimizing adult populations. Furthermore, in recent years, control of adult mosquitoes has become increasingly difficult because of insecticide resistance and behavioral changes such as the avoidance of mosquito vectors to residual insecticides [3–5]. It is easier and more efficient to control the delicate larvae that are relatively immobile and more concentrated, having not yet left their aquatic breeding sites [6, 7]. Moreover, there has been increasing documentation of resistance of larval populations of anopheline mosquitoes, malaria vectors, to one or more of the main groups of conventional synthetic insecticides, that is, organochlorines, organophosphates, carbamates, and pyrethroids [8–14]. One of the most promising ways of minimizing development of insecticide resistance and reducing negative impacts to human and other living organisms and the environment is applying nonchemical materials, that is, biopesticides that do not confer cross-resistance to current insecticides and are naturally biodegradable into nontoxic [15–18].

Insecticides of botanical origin are attractive alternatives because they contained rich sources and various bioactive compounds, many of which are selective and have little or no harmful effect on nontarget organisms and the environment [19, 20]. Furthermore, the complex and variable mixtures of bioactive constituents with different modes of action may lessen the chance of resistance in mosquito populations [21]. Recently, essential oils have received considerable attention as a potentially useful bioactive insecticide, with their low mammalian toxicity and rapid degradability in the environment [22]. Larvicidal activities have been demonstrated in many plant oils such as neem, basil, cinnamon, citronella, camphor, eucalyptus, lemon, and pine [15, 23–26]. Combined formulations of different essential oils, which have more active substances than individuals, have also been investigated as larvicides, and some mixtures were found to be more effective than neem (*Azadirachta indica*) extract [27, 28]. Neem and neem-based products have been widely acknowledged and currently available as the prominent biopesticides because of their pesticidal potential with larvicidal and growth regulating activity. Nevertheless, if they are used indiscriminately, they may induce resistance in the pests and can be rendered ineffective within a few years [29]. Thus, the finding of new botanical pesticides, particular combinations of two or more toxicants with different mechanisms of action, is the need of the hour. However, a lot more work has been done on the coupled effects of synthetic-synthetic pesticides than plant-synthetic and plant-plant pesticide combinations [30]. Furthermore, most studies on the combined insecticidal efficacy of phytochemical-mixed formulations have been conducted on agricultural pests rather than pests of medical importance [31]. The present study was undertaken, therefore, to investigate the chemical composition and larvicidal efficacy of indigenous plant-derived essential oils and their combinations against *A. cracens*; a potential vector of malaria, with the aim of developing essential oil-mixed larvicides as supplementary and complementary measures for the management of malaria vectors.

2. Materials and Methods

2.1. Plant Materials. Ten plant species belonging to six families, Cyperaceae, Myristicaceae, Piperaceae, Rutacea, Umbelliferae, and Zingiberaceae, which mostly consist of botanicals with promising bioactivity against mosquitoes [31, 32], were selected for screening larvicidal activity against *A. cracens*. The plant materials (Table 1) were collected from natural habitats or commercially obtained from medicinal herb suppliers in Chiang Mai province. The herbarium specimen of each plant was identified and authenticated by botanists and plant taxonomists from the Department of Biology, Faculty of Science and the Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Thailand. The voucher specimens were numbered and deposited at the Department of Parasitology, Faculty of Medicine, Chiang Mai University.

2.2. Extraction of the Essential Oils. The plant materials utilized for extracting the essential oil were shade-dried at the environmental temperature (27–36°C) and then separately ground by an electrical blender. Dried and coarsely ground plants were extracted individually by steam distillation at 100°C for at least 3 hours to obtain the ethereal oil. The oil layer was separated from the aqueous phase, filtrated and dried over anhydrous sodium sulfate (Na_2So_4) to remove traces of moisture. Physical characteristics of the oil were recorded and the percentage yield was averaged over three experiments and calculated according to dry weight of the plant materials. The resulting essential oils were subsequently stored in an amber-colored bottle under refrigeration (4°C) until analysis for chemical compositions and larvicidal activity.

2.3. Mosquito Colony Handling. The colony of *A. cracens* [33], formerly *A. dirus* (species B), was obtained originally from the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. The free-mating populations of this mosquito had been established for more than 2 decades in the insectary of Department of Parasitology, Faculty of Medicine, Chiang Mai University [34]. The mosquito colony was maintained continually without exposure to any pathogens and insecticides under a constant laboratory condition at temperature of 27 ± 2°C and 70–80% relative humidity under a photoperiod of 12 : 12 hours (light/dark). Adults were incessantly provided with 10% sucrose and 5% multivitamin syrup solution in a small bottle with a cotton wick. Rats were supplied as a blood source for egg production of adult females. Eggs were collected and kept in plastic cups lining with moistened filter paper. Larvae were reared in plastic trays on the meal of powdered fish food. Freshly molted larvae (L_4) of *A. cracens* taken from the mass culture were available continuously for the mosquito larvicidal experiments.

2.4. Preliminary Screening for Larvicidal Activity of Essential Oils. Preliminary screening of essential oils derived from various parts of ten plants was carried out at the high concentration, 100 ppm, to check for larvicidal activity. Essential

Table 1: Physical characteristics and percentage yields (% Yield) of essential oils derived from ten plant species.

Family and botanical name (reference number)	English name	Part used	Physical characteristics			% Yield
			Color	Odor	Density (g/mL)	
Cyperaceae						
Cyperus rotundus Linn. (PARA-CY-001/1)	Nut grass	Tuber	Golden yellow	Nut grass-like	0.95	0.42
Myristicaceae						
Myristica fragrans Houtt. (PARA-MY-001/1)	Nutmeg	Mace	Light yellow	Nutmeg-like	0.96	3.41
Piperaceae						
Piper nigrum Linn. (PARA-PI-004/1)	Black pepper	Fruit	Clear	Pepper-like	0.90	0.39
Piper longum Linn. (PARA-PI-001/5)	Long pepper	Fruit	Light yellow	Pepper-like	0.87	0.64
Piper sarmentosum Roxb. (PARA-PI-003/2)	Wild betel	Leaf and stem	Brown	Pepper-like	0.91	0.31
Rutacea						
Zanthoxylum piperitum DC. (PARA-ZA-002/4)	Japanese Prickly Ash	Fruit	Pale yellow	Orange-like	0.74	0.34
Umbelliferae						
Coriandrum sativum Linn. (PARA-CO-002/2)	Coriander	Fruit	Pale yellow	Bug-like	0.86	0.97
Foeniculum vulgare Mill. (PARA-FO-001/3)	Fennel	Fruit	Pale yellow	Anise-like	0.89	0.57
Zingiberaceae						
Amomum uliginosum Koenig (PARA-AM-002/2)	Cardamom	Rhizome	Light yellow	Camphor-like	0.92	0.95
Curcuma longa Linn. (PARA-CU-005/1)	Turmeric	Rhizome	Pale yellow	Ginger-like	0.81	0.56

oil was individually dissolved in a nontoxic emulsifying agent, dimethylsulphoxide (DMSO). Groups of 25 early 4th instar larvae of *A. cracens* were selected and then exposed to the test concentration containing 249 mL of distilled water and 1 mL of essential oil-DMSO solution. Bioassays were set up according to a slightly modified version of the standard WHO larval susceptibility test methods [35] under the similar conditions used for rearing. Four replicates were maintained for the individual oil along with the concurrent control and untreated groups. A control group received DMSO-distilled water, while the untreated one was maintained in distilled water only. Mortalities of treated larvae were determined after an exposure period of 24 hours. The larvae were considered dead if they were unable to move or respond when stimulated by probing with a blunt dissecting needle. Moribund larvae were those incapable of rising to the surface of the water or showing a characteristic diving reaction when the water was disturbed. The moribund and dead larvae in each test were combined and expressed as percentage mortalities, which were corrected for control mortality using Abbott's formula [36].

2.5. Dose-Response Bioassay. Based on the initially larvicidal screening results, the promising oils, which produced 95–100% larval mortalities, were subjected to a dose-mortality response bioassay. Plant oil-DMSO solutions were prepared into different concentrations with distilled water in the range of 10 to 80 ppm, depending on the plant species. The dose response bioassays were carried out as in the screening protocol previously described. Tests were conducted using four batches of 25 larvae with the final total number of 100 larvae for each concentration. Every bioassay was replicated four times with mosquitoes from different rearing batches. The percentage mortality was reported from the average of four replicates.

2.6. Essential Oil-Mixed Formulation Experiment. Combinations comprising various mixing ratios of pairs of the most effective and the other oils established from the dose-response experiments were evaluated against *A. cracens*, as previously done, to determine whether these mixtures increase larvicidal efficacy compared with the constituted oil

Table 2: Chemical constituents of essential oils derived from five plants.

No.	Constituent	RT	Percentage composition (%)				
			P. sarmentosum	*F. vulgare*	*C. longa*	*M. fragrans*	*Z. piperitum*
1	α-Thujene	7.12				0.67	
2	α-Pinene	7.27				0.98	1.40
3	Sabinene	8.13				14.25	6.13
4	β-Pinene	8.20				0.52	
5	β-Myrcene	8.48				1.07	3.08
6	Phellandrene	8.76				0.81	
7	α-Terpinene	8.99				1.11	
8	p-Cymene	9.15			0.87	1.52	4.42
9	α-Limonene	9.23		2.07			12.03
10	β-Terpinene	9.24				16.13	
11	1,8-Cineole	9.29			0.91	0.66	21.27
12	γ-Terpinene	9.79				2.66	
13	p-Mentha-1,4-diene	9.97				0.72	
14	α-Terpinolene	10.33				0.65	
15	Fenchone	10.35		8.90			
16	Linalool	10.52				0.67	6.10
17	Thujene	10.92					0.83
18	1-Terpinen-4-ol	11.85				6.56	4.74
19	2-Allyltoluene	11.97					0.86
20	Cryptone	12.01					3.15
21	α-Terpineol	12.06				0.57	5.48
22	Estragole	12.16		5.70			1.54
23	Cuminal	12.83					0.68
24	3-Carene	12.98					2.96
25	4-Anisaldehyde	13.04		16.29			
26	Piperitone	13.05					7.31
27	Anethole	13.50		63.00			
28	Safrole	13.56				46.60	
29	Limonene	14.36					8.50
30	Geraniol	14.76					1.21
31	α-Copaene	14.78	3.77				
32	p-Acetonylanisole	14.84		1.16			
33	β-Elemene	14.98	0.70				
34	Methyleugenol	15.06				2.80	
35	β-Caryophyllene	15.40	7.38		1.58		
36	α-Humulene	15.84	0.80				
37	γ-Muurolene	16.09	0.48				
38	α-Curcumene	16.12			9.53		
39	*d*-Germacrene	16.19	1.22				
40	β-Selinene	16.26	1.56				
41	Zingiberene	16.27			3.93		
42	α-Selinene	16.37	1.56				
43	β-Bisabolene	16.44			2.25		
44	α-Amorphene	16.58					0.70
45	β-Sesquiphellandrene	16.64			8.55		
46	Croweacin	16.67	71.01				
47	Elemicin	16.96	2.47			1.03	
48	Farnesol	17.07	0.44				

Table 2: Continued.

No.	Constituent	RT	Percentage composition (%)				
			P. sarmentosum	*F. vulgare*	*C. longa*	*M. fragrans*	*Z. piperitum*
49	Caryophyllene oxide	17.46					1.45
50	Aromadendrene	17.47	0.77				
51	α-Cedrene	17.71			0.75		
52	γ-Gurjunene	18.24	0.61				
53	β-Maaliene	18.26	0.52				
54	ar-Turmerone	18.32			30.19		
55	Tumerone	18.36			19.02		
56	Brevifolin	18.42					6.15
57	Curlone	18.73			13.30		
	Total identified		93.29	97.12	90.88	99.98	99.99

RT: Retention time (min).

alone. The combined action of essential oils individually in the oil-mixed formulation was decided on the basis of LC_{50} value of each oil and cotoxicity coefficient (CTC) of mixtures.

2.7. GC/MS Analysis of the Effective Plant Oils. GC/MS analysis was carried out to identify the chemical constituents of the effective plant oils. Essential oils demonstrating highly larvicidal activity against *A. cracens* were subjected to analysis by using an Agilent 7890 GC system 5975 MSD, performing under the following conditions: carrier gas helium (1.0 mL/min), diluter dichloromethane (1/10, v/v), and injector temperatures 250°C using a capillary column (HP5MS 30 m × 0.25 mm, ID × 0.25 μm film thickness). The sample (0.5 μL) was injected neat with a split ratio of 250 : 1. The initial oven temperature was 50°C (hold 4 min) with a 10°C/min dynamic ramp to 250°C. Identification of oil constituents was made by comparison of mass spectra of each peak with those of authentic samples in a mass spectra Wiley 8N08 GC/MS library. Relative percentage amount of the identified compound was computed from a total ion chromatogram (TIC).

2.8. Data Management and Statistical Analysis. In all cases where deaths had occurred in the control experiment, the mortality data was corrected by Abbott's formula [36] and then determined by computerized probit analysis (Harvard Programming; Hg1, 2). Larvicidal activity was reported as LC_{50}, LC_{95}, and LC_{99} values along with corresponding 95% confidence intervals (CI), representing the concentrations that induced 50, 95, and 99% mortality, respectively. Values were considered to be significantly different ($P \leq 0.05$) if CI were nonoverlapping. A cotoxicity coefficient (CTC) for mixed formulation experiments, which is based on the lethal concentration and the proportion of each oil component in the mixture, was used to determine their responses: similar, synergism, and antagonism. When CTC of a mixture is 100, it indicates the probability of similar (additive) action. If the mixture gives a CTC greater than 100, it indicates a synergistic action. On the other hand, when a mixture gives a CTC less than 100, it is considered antagonism [37–39]. If a mixture (M) formulation of two oils (A and B), and both components have LC_{50}, then the following formulas are used (A serving as standard):

$$\begin{aligned}
&\text{Toxicity index (TI) of A} = 100,\\
&\text{Toxicity index (TI) of B} = \frac{LC_{50}\text{ of A}}{LC_{50}\text{ of B}} \times 100,\\
&\text{Actual TI of M} = \frac{LC_{50}\text{ of A}}{LC_{50}\text{ of M}} \times 100,\\
&\text{Theoretical TI of M} = \text{TI of A} \times \%\text{ of A in M}\\
&\qquad\qquad + \text{TI of B} \times \%\text{ of B in M},\\
&\text{Cotoxicity coefficient (CTC)}\\
&\qquad\qquad = \frac{\text{Actual TI of M}}{\text{Theoretical TI of M}} \times 100.
\end{aligned} \tag{1}$$

If one component of the mixture alone (e.g., B) causes low mortality at all doses (<20%), then CTC of the mixture was calculated by the formula:

$$\text{Cotoxicity coefficient} = \frac{LC_{50}\text{ of A alone}}{LC_{50}\text{ of A in the mixture}} \times 100. \tag{2}$$

3. Results and Discussion

Steam distillation of ten medicinal plants yielded from 0.31 to 3.41% (v/w) essential oils according to dry weight (Table 1). The highest oil content was found in *M. fragrans* (3.41%), followed by *C. sativum* (0.97%), *A. uliginosum* (0.95%), *P. longum* (0.64%), *F. vulgare* (0.57%), *C. longa* (0.56%), *C. rotundus* (0.42%), *P. nigrum* (0.39%), *Z. piperitum* (0.34%), and *P. sarmentosum* (0.31%). The physical and organoleptic properties of these oils presented in Table 1 demonstrate the slight differences in appearance, color, odor, and density. These volatile oils had a characteristic smell and were clear, yellow, and brown liquids that were less dense than water.

In the larvicidal screening experiment, of the essential oils initially tested at a concentration of 100 ppm, the oils

Table 3: Larvicidal activity of plant-derived essential oils against the 4th instar larvae of *A. cracens*.

Concentration of plant oil (ppm)	% Mortality (mean ± SE)	Larvicidal activity (95% CI, ppm)			Slope values ± SE
		LC_{50}	LC_{95}	LC_{99}	
Piper sarmentosum					
12.7	9.25 ± 3.30				
14.6	23.50 ± 1.29				
16.4	53.75 ± 5.44	16.03 (15.51–16.54)	20.64 (20.01–21.86)	22.91 (22.12–24.66)	14.9920 ± 0.5669
18.2	79.50 ± 2.65				
20.0	94.50 ± 3.11				
Foeniculum vulgare					
22.3	6.50 ± 1.73				
26.7	12.00 ± 4.08				
31.2	41.00 ± 11.83	32.77 (31.44–34.11)	46.56 (44.84–49.83)	53.86 (51.67–58.61)	10.7846 ± 0.3708
35.6	64.75 ± 6.65				
40.1	82.00 ± 3.74				
44.5	94.25 ± 4.99				
Curcuma longa					
20.3	12.50 ± 2.08				
24.3	17.50 ± 3.00				
28.4	23.50 ± 2.65				
32.4	31.00 ± 4.40	33.61 (29.43–39.15)	56.49 (59.66–82.13)	70.04 (79.34–112.48)	7.2941 ± 0.2698
36.5	47.25 ± 5.44				
40.5	83.75 ± 0.96				
44.6	90.50 ± 1.29				
Myristica fragrans					
28.8	10.00 ± 1.15				
33.6	17.75 ± 2.50				
38.4	34.75 ± 4.19	40.00 (37.33–43.32)	56.56 (55.76–67.70)	65.28 (65.35–81.90)	10.9335 ± 0.4652
43.2	63.75 ± 3.86				
48.0	85.75 ± 3.09				
Zanthoxylum piperitum					
51.8	11.75 ± 1.71				
55.5	29.50 ± 5.26				
59.2	35.25 ± 9.22				
62.9	43.50 ± 2.08	63.17 (61.90–64.50)	85.01 (82.59–89.33)	96.13 (92.67–102.67)	12.7574 ± 0.5292
66.6	61.00 ± 4.32				
70.3	73.50 ± 2.08				
74.0	83.00 ± 3.77				

derived from five plants, including *P. nigrum*, *A. uliginosum*, *C. sativum*, *P. longum*, and *C. rotundus* produced no or low larval mortality of 0, 4, 8, 36, and 52%, respectively. No larval mortality was observed in the control and untreated groups. The other oils, including *P. sarmentosum*, *F. vulgare*, *C. longa*, *M. fragrans*, and *Z. piperitum* demonstrated promising efficacy with larval mortality of 100, 100, 100, 100, and 96%, respectively. These five plants were then selected for further experiments, including chemical analysis, dose-response larvicidal experiments, and combination-based bioassays for quantifying their toxicity.

Results of phytochemical analysis of the essential oils with promising larvicidal activity are displayed in Table 2. A total of 57 compounds were identified from five essential oils, including *P. sarmentosum*, *F. vulgare*, *C. longa*, *M. fragrans*, and *Z. piperitum*, representing 90.88–99.99% of the oil obtained. The oil derived from leaf and stem of *P. sarmentosum* contained 14 identified compounds, amounting to 93.29% of the whole oil with croweacin (71.01%) as the chief constituent, together with minor amounts of β-caryophyllene (7.38%), α-copaene (3.77%), and elemicin (2.47%). In the fruit oil of *F. vulgare*, 6 compounds

Table 4: Larvicidal activity and cotoxicity coefficient (CTC) of five essential oils and *P. sarmentosum*-combined oil formulations against the 4th instar larvae of *A. cracens*.

Essential oil	Combination of essential oil	LC_{50} (95% CI, ppm)	Slope values ± SE	Cotoxicity coefficient (CTC)	Effect
P. sarmentosum (P)	P 100%	16.03 (15.51–16.54)	14.9920 ± 0.5669	—	—
F. vulgare (F)	F 100%	32.77 (31.44–34.11)	10.7846 ± 0.3708	—	—
C. longa (C)	C 100%	33.61 (29.43–39.15)	7.2941 ± 0.2698	—	—
M. fragrans (M)	M 100%	40.00 (37.33–43.32)	10.9335 ± 0.4652	—	—
Z. piperitum (Z)	Z 100%	63.17 (61.90–64.50)	12.7574 ± 0.5292	—	—
P + F	P 25% : F 75%	28.60 (28.37–28.83)	17.0907 ± 0.8318	90.8595	Antagonism
	P 50% : F 50%	27.29 (26.09–28.43)	15.0440 ± 0.6262	78.8890	Antagonism
	P 75% : F 25%	18.32 (17.65–18.99)	9.1834 ± 0.4084	100.3105	Synergism
P + C	P 25% : C 75%	27.10 (25.04–28.80)	6.8012 ± 0.2937	97.3354	Antagonism
	P 50% : C 50%	22.08 (21.51–22.61)	14.9567 ± 0.5742	98.3108	Antagonism
	P 75% : C 25%	16.81 (16.59–17.03)	10.8101 ± 0.4357	109.7055	Synergism
P + M	P 25% : M 75%	35.72 (33.51–37.74)	9.7333 ± 0.4288	81.5108	Antagonism
	P 50% : M 50%	28.51 (27.48–29.67)	14.7402 ± 0.7607	80.2797	Antagonism
	P 75% : M 25%	18.18 (17.69–18.64)	12.4666 ± 0.4585	103.7110	Synergism
P + Z	P 25% : Z 75%	41.40 (40.45–42.19)	32.5982 ± 1.4760	87.9356	Antagonism
	P 50% : Z 50%	29.40 (28.33–30.59)	19.9294 ± 0.7629	86.9765	Antagonism
	P 75% : Z 25%	17.99 (16.31–20.45)	6.8213 ± 0.3053	109.5410	Synergism

were identified, representing 97.12% of the oils obtained. Compounds in this oil comprised mostly anethole (63.00%), followed by 4-anisaldehyde (16.29%), with minor contents of fenchone (8.90%), estragole (5.70%), and α-limonene (2.07%). For *C. longa* rhizome oil, 11 compounds were identified, corresponding to 90.88% of the total oil. The major components were ar-turmerone (30.19%), tumerone (19.02%), and curlone (13.30%), whereas α-curcumene (9.53%) and β-sesquiphellandrene (8.55%) were seen as minor constituents. The mace oil of *M. fragrans* demonstrated the presence of 19 compounds, accounting for 99.98% of the whole oil with safrole (46.60%) as the principal constituents, followed by β-terpinene (16.13%), sabinene (14.25%), and 1-terpinen-4-ol (6.56%). Twenty one compounds constituting 99.99% of all the volatile compositions were characterized from *Z. piperitum* fruit oil. The main chemical compounds identified were 1,8-cineole (21.27%) and α-limonene (12.03%), followed by minor quantities of limonene (8.50%), piperitone (7.31%), brevifolin (6.15%), sabinene (6.13%), and linalool (6.10%).

In the dose-response larvicidal assessment, all the oils examined exhibited a promising larvicidal efficacy on larvae of *A. cracens* with dose dependent and different performances among plant species. The strongest larvicidal potential was established from *P. sarmentosum*, followed by *F. vulgare*, *C. longa*, *M. fragrans*, and *Z. piperitum*, with LC_{50} values of 16.03, 32.77, 33.61, 40.00, and 63.17 ppm, respectively (Table 3). Although bioactivity of the essential oil results from interaction among structural components, particularly the major constituents, the other compounds, even trace elements, can also have a vital function; this is due to coupled effects, additive action between chemical classes and synergy or antagonism [40, 41]. Further investigations of comparative toxicity of chemical constituents derived from these plants, either individually or in selected blends, are necessary for identifying components contributing to the observed larvicidal action. Bekele and Hassanali [42] investigated the lethal toxicity of major components derived from essential oils of *Ocimum kilimandscharicum* (camphor, limonene, 4-terpeneol, 1,8-cineole, camphene, and *t*-caryophyllene) and *Ocimum kenyense* (methyl chavicol, ethyl isovalerate, α-humulene, 1,8-cineole, and isoeugenol) against two postharvest insect pests, *Sitophilus zeamais* and *Rhyzopertha dominica*. They discovered that a major compound of *O. kilimandscharicum* was largely responsible for the toxic effect against *R. dominica*. However, the results with the other treatments indicated that the toxic action of the essential oils was due to the combined effects of different components, either with or without significant individual toxic action of their own against the insects. Some of these compounds such as 1,8-cineole, limonene, and humulene are presented in the plant oils tested in this study and also found in other plants with biological activity against various insect species [43–45].

Generally, individual botanical insecticides are slow acting, time consuming, and active only at high concentration, which makes them impractical and uneconomical for field application [27, 46]. Phytochemical-combined formulations, which not only improve activity, but also decrease the needed dose, are therefore considered very advantageous in vector control program. The importance of proper selection of plant extracts as synergists in mixed formulations with different botanicals is being increasingly recognized in mosquito management [30]. Mixtures of more than one insecticide

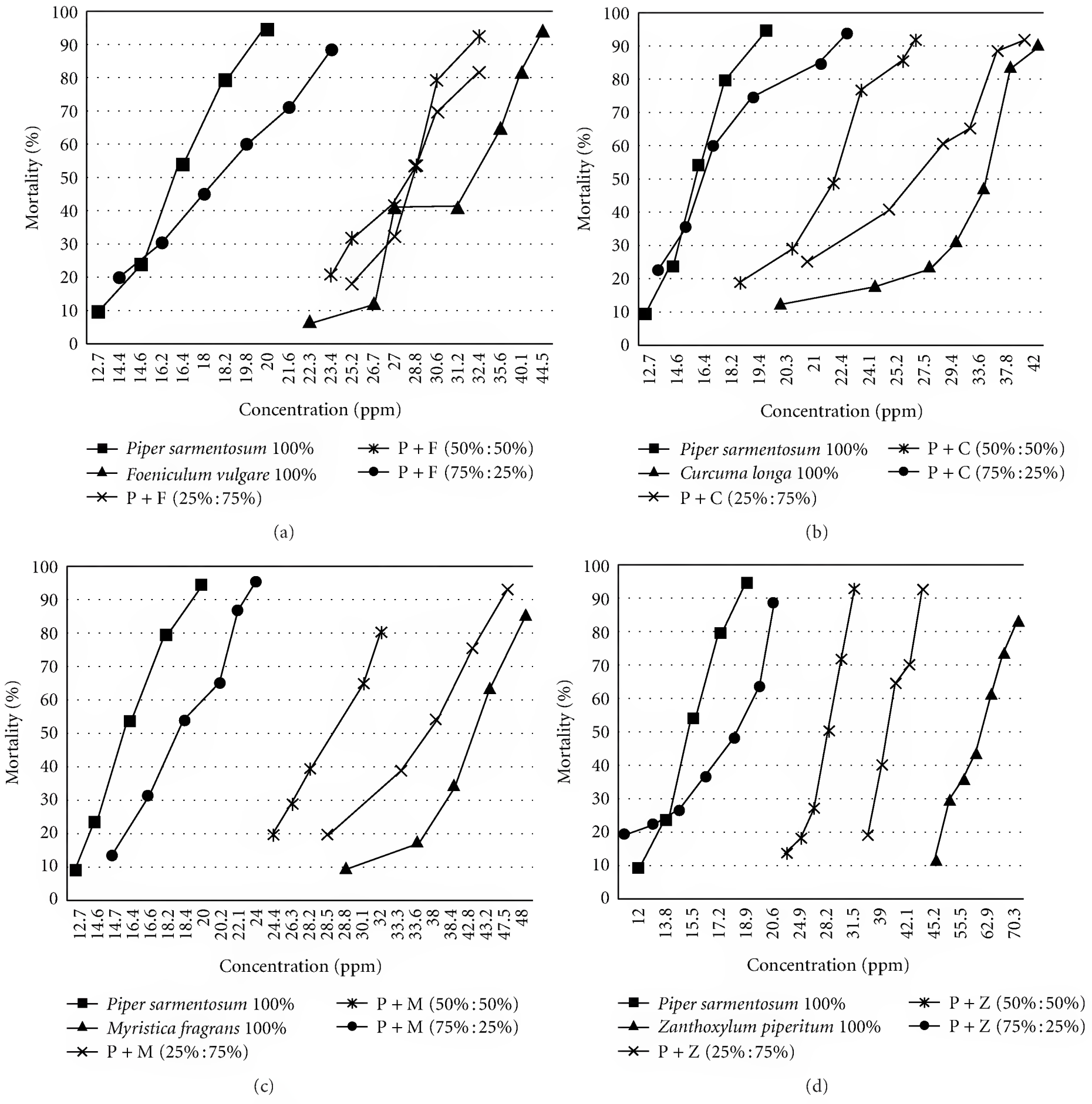

FIGURE 1: Larvicidal activity of combined formulations between *P. sarmentosum* (P) oil and the other plant oils: (a) *F. vulgare* (F), (b) *C. longa* (C), (c) *M. fragrans* (M), and (d) *Z. piperitum* (Z) against the 4th instar larvae of *A. cracens*.

with different modes of actions are proving to be effective and recommended for integrated resistance management in some insect pests [47–50]. In this study, comparative evaluation of the larvicidal efficacy of combinations between *P. sarmentosum*, the most efficient oil, and the others was carried out and the results are demonstrated in Figure 1 and Table 4. It was found that the addition of *P. sarmentosum* oil to the other individual oils affected the larvicidal activity, leading to increasing mortality of *A. cracens* larvae in all trials. The binary mixtures of oils of *P. sarmentosum* and the others, including *F. vulgare*, *C. longa*, *M. fragrans*, and *Z. piperitum* at the ratios of 25% : 75%, 50% : 50%, and 75% : 25% showed remarkably reduced LC_{50} values, ranging from 18.32–28.60, 16.81–27.10, 18.18–35.72, and 17.99–41.40 ppm, respectively. The cotoxicity coefficient (CTC) determined from these LC_{50} values were ranged from 78.8890–100.3105, 97.3354–109.7055, 80.2797–103.7110, and 86.9765–109.5410, respectively. The combined effect of *P. sarmentosum* and the other oils at the highest ratio (75% : 25%) possessed synergistic activity with a value CTC (relative to LC_{50}) greater than 100. However, all mixtures at the lower ratios (25% : 75% and 50% : 50%) exhibited antagonistic action with a CTC value lower than 100.

In the present study, combinations of *P. sarmentosum* and the other oils exhibited better larvicidal activity than most independent oils. Although the effect at the lower ratios

(25% : 75% and 50% : 50%) was relatively moderate, the larvicidal activity was significantly improved when the mixtures (75% : 25% ratio) contained higher amount of *P. sarmentosum*. Of special interest is in the case of *C. longa*, *Z. piperitum*, and *M. fragrans* oils, which have lower larvicidal efficacy than that of *F. vulgare*; addition of *P. sarmentosum* in these three oils at the highest ratio (75% : 25%) gave a mixture that is more active (LC_{50} = 16.81, 17.99, and 18.18 ppm, resp.) than that of *P. sarmentosum-F. vulgare* mixed formulation (LC_{50} =18.32 ppm). From these findings, it was suggested that combinations between *P. sarmentosum* and the other oils in the appropriate varieties and proportions are beneficial in enhancing larvicidal toxicity toward anopheline mosquitoes. In addition, in the case of *Z. piperitum* oil (2.71 USD/mL), which is approximately three times more expensive than *P. sarmentosum* oil (0.94 USD/mL), combined formulations of these two oils provided not only better efficacy but also lower cost. The synergistic larvicidal activity of combinations between two plant extracts, *Hyptis suaveolens* and *Lantana camara*, was previously reported by Tanprasit [28]. It was revealed that the mixture of *H. suaveolens* and *L. camara* (LC_{50} = 14.04%) possessed significantly higher larvicidal activity against *Aedes aegypti* than those of the individual substances, *H. suaveolens* (LC_{50} = 20.24%) and *L. camara* (LC_{50} = 74.44%). The individual and combined efficacy of *Annona squamosa* and *Pongamia glabra* extracts against three mosquito vectors, *Culex quinquefasciatus*, *Anopheles stephensi*, and *A. aegypti*, compared to that of *A. indica* was investigated by George and Vincent [27]. It was found that *P. glabra* has a greater larvicidal effect than that of *A. squamosa*, and all of their combined formulations exhibited significantly greater effect than those of independent extracts. Furthermore, the most effective mixture of these plant extracts (LC_{50} = 28.804 ppm) was found to be more effective than the prominent biopesticide, *A. indica* (neem) extract (LC_{50} = 45.120 ppm). Singha et al. [51] reported the synergistic effect of *Croton caudatus* (fruit) and *Tiliacora acuminate* (flower) extracts against filarial vector, *C. quinquefasciatus*. The combined formulation of *C. caudatus* and *T. acuminate* exhibited good bioactive potentiality against *C. quinquefasciatus* larvae due to synergism of plant extracts. These findings correspond to those of this study, which presents an insight into the high possibility of developing new mosquitocides from combinations of different essential oils or phytochemicals, generating synergism. Remarkably better performance of *P. sarmentosum* in the essential oil-mixed formulation experiment herein suggests that it may have good potential to be an alternative synergist in efficient mixtures of control agents. This performance may achieve satisfactory levels of efficacy, economic benefit, and ecological friendliness and minimize the development of resistance in the vector population.

Acknowledgments

This work is supported by the Faculty of Medicine Research Fund, Faculty of Medicine, Chiang Mai University, Thailand. The authors are grateful to Dr. Arunothai Jampeetong, a Taxonomist and Botanist at the Department of Biology, Faculty of Science, and Assistant Professor Dr. Sunee Chansakaow, a Taxonomist at the Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Thailand, for their kindness in identification of the plant samples.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 850893, 7 pages
doi:10.1155/2012/850893

Research Article

First Record of *Pyramica epinotalis* (Hymenoptera: Formicidae) for the United States

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Received 29 March 2012; Accepted 10 April 2012

Academic Editor: David Roubik

Pyramica epinotalis is an arboreal dacetine ant previously known only from Brazil, Costa Rica, Ecuador, and southern Mexico. Here we report the first records of *P. epinotalis* for the United States. Collections were made in three parishes across southern Louisiana in cypress-tupelo swamps using floating pitfall traps placed in floating vegetation and arboreal pitfall traps placed on trunks and limbs of three wetland tree species. One additional specimen of this species was collected in Highlands County, Florida. Based on collections of specimens in Louisiana, including multiple dealate females at different localities, *P. epinotalis* appears to be well established in this state. We discuss the design and implementation of modified arboreal pitfall traps that were instrumental in this discovery.

1. Introduction

The tribe Dacetini (Hymenoptera: Formicidae) is composed of small, cryptic, predatory ants that typically occur in soil and/or leaf litter where they feed on various minute arthropods [1, 2]. Species in this group show great diversity in predatory strategies, which is reflected in the marked differentiation between species groups. With their unique-looking body types and head shapes that are variously adorned with bizarre station, elongate mandibles with uniquely arrayed dentition, and as-yet-unexplained cuticular outgrowths called spongiform tissue, members of this group are among the most unusual in the ant world. This large and diverse tribe includes more than 900 described species worldwide, of which 327 are in the genus *Pyramica* [3]. Primarily considered a tropical group, only 41 species of *Pyramica* have been reported from the USA. Thirty-seven of these species occur in the southeastern United States [4]. Five species of the related *Strumigenys* are known from the same region [5]. The relatively high density of dacetine species in the Southeast is likely due to the humid, subtropical climate and mild winters typical of the region and the availability of large continuous tracts of forested habitats, which appear to facilitate establishment of these species' colonies.

Currently, nine introduced dacetine species are known from the southeastern USA including *Pyramica eggersi* (Emery) (Florida), *P. gundlachi* (Roger) (Florida), *P. hexamera* (Brown) (Alabama, Florida, Louisiana, and Mississippi), *P. margaritae* (Emery) (Alabama, Florida, Georgia, Louisiana, and Mississippi), *P. membranifera* (Emery) (Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, North Carolina, and South Carolina), *Strumigenys emmae* (Emery) (Florida), *S. lanuginosa* Wheeler (Florida), *S. rogeri* Emery (Florida), and *S. silvestrii* Emery (Alabama, Florida, Georgia, Louisiana, and Mississippi) [6]. *Pyramica subnuda* MacGown and Hill, which is in the *schulzi* species group, may also be introduced despite the fact that it was described from a specimen collected in Mississippi as other members of this group are tropical [4]. Here, we present records of another introduced dacetine ant from South America, *P. epinotalis* (Weber), which also belongs to the *schulzi* species group.

2. Taxonomy and Identification

Weber described *Strumigenys (Cephaloxys) studiosi* subsp. *epinotalis* (= *P. epinotalis*) in 1934 from specimens collected by G. C. Wheeler from Costa Rica, Prov. Limon, Estrella Valley, Talia Farm, 18.vi.1924 [7]. *Strumigenys* (*Cephaloxys*) *skwarrae,* a synonym of *P. epinotalis*, was described by W. M. Wheeler in 1934 from specimens collected in *Tillandsia streptophylla* Scheidweiler (Bromeliaceae) in Tlacocintla, Mexico by E. Skwarra in 1929 [8]. In 1953, Brown synonymized *S. skwarrae* with *S. epinotalis* and transferred it to *Smithistruma* [9]. Bolton later moved the species to *Pyramica* in 1999 [10], Baroni Urbani and De Andrade synonymized *Pyramica* with *Strumigenys* in 2007 [11], and Bolton and Alpert reconfirmed *Pyramica* as a valid genus in 2011 [12].

2.1. Worker (Figures 1(a)–1(c)) (Description Modified from Bolton [13]). Total length 1.9–2.1 mm. Head wedge-shaped. Color yellowish-brown, appendages only slightly paler. Entire head including clypeus with reticulate-punctate sculpture. Mandibles subtriangular, lacking diastema; nine acute teeth present following basal lamella; third tooth from basal lamella spiniform, elongate and distinctly longer than other teeth, with subsequent teeth alternating in length with the fifth being longer than the fourth and the seventh being longer than the sixth, and the remaining two teeth smaller and blunter. Clypeus somewhat pentagonal shaped, narrowing anteriorly, and with anterior margin slightly convex. Dorsum of clypeus with numerous clavate hairs directed anteriorly or away from midline of clypeus; clypeal margin with similarly shaped clavate hairs all curving anteriorly toward midline of clypeus; remainder of head with slightly larger clavate to spoon-shaped hairs that curve toward midline of head; elongate flagelliform cephalic hairs absent; and leading edge of scape with a row of elongate, curved hairs, all of which curve toward the base of the scape or are directed downward. Eye large with 5–7 ommatidia in longest diameter.

Mesosoma, including sides, and petiole with distinct reticulate-punctate sculpture; postpetiole disc lacking sculpture, smooth. Pronotum with an arched transverse ridge with rowed, erect spoon-shaped hairs; pronotal humeral hairs absent; mesonotum with appressed spoon-shaped hairs. Propodeal spines somewhat short and dentiform, directed upward; propodeal declivity bordered by a high arched lamella on each side. Petiole with longitudinal spongiform crest ventrally and fan-shaped, spongiform bodies present posteriorly; elongate, spoon-shaped hairs present dorso-posteriorly. Postpetiole with large, spongiform mass ventrally, but becoming a lamina-like structure posteriorly; with elongate, spoon-shaped hairs present dorsoposteriorly. First gastral tergite smooth and shining except for basigastral costulae, which are distinct and extend to at least the basal quarter to third of the length of the tergite. Several to numerous suberect to erect, elongate, thickened hairs present on first tergite.

2.2. Alate Female (Figures 1(d) and 1(e)). Similar to worker, but larger (total length approximately 2.5 mm), ocelli present, mesosoma enlarged with typical modifications for flight muscles, wings present, and katepisternum mostly lacking sculpture.

This species is easily differentiated from other species known from the USA by the combination of having the third tooth (from basal lamella) on mandible longer than the other teeth, the mesosoma of the worker being completely reticulate-punctate, having a curved row of spoon-shaped hairs on the pronotal dorsum, a distinct propodeal lamella, a ventral spongiform crest beneath the petiole, and fan-shaped patches of spongiform tissue on the petiole and postpetiole. Currently, the only other species reported from the USA with which *P. epinotalis* might be confused is *P. margaritae*, another introduced species in the *schulzi* group. *Pyramica margaritae* is the only other species known to occur in the United States that has sculpture on the entire side of the mesosoma; however, *P. margaritae* lacks a curved row of spoon-shaped hairs on the pronotal dorsum, has much longer propodeal spines, lacks a propodeal lamella, lacks spongiform bodies beneath the petiole, and has reduced spongiform tissue present beneath the postpetiole.

3. Natural History

Although the vast majority of dacetine ants nest in soil and leaf litter, members of the *schulzi* species group are typically associated with plants, and several species have been recorded from epiphytes or plant cavities [13]. Many species in this group also differ in that workers have enlarged compound eyes, as compared with their epigeic and hypogeic relatives. Similar to most members of this group, *P. epinotalis* also has enlarged eyes and is thought to be an arboreal species. Weber described this species in 1934 from specimens collected by George Wheeler in 1924 in an Atlantic slope wet forest in Costa Rica, but he did not indicate whether the ants were collected arboreally or in litter [7]. Collecting in the same region years later, Longino reported that multiple litter samples from near the type locality did not yield specimens of this species, which suggests that perhaps this species might be arboreal [14]. In 1934, Wheeler reported that Dr. Skwarra discovered four colonies of this species (reported as *Strumigenys skwarrae* Wheeler) in *Tillandsia streptophylla* at two localities in Mexico in 1929 [8]. Bolton [13] reported that collections of this species were made in Mexico by Dressler and by Dejean, both of whom worked with epiphytes, which implies that their specimens were also from epiphytes. More recently, Rider reported collections of this species in the canopy in Ecuador, which further validates its status as an arboreal species [15].

4. Methods

A single alate female was collected by Mark Deyrup on 14 August 2009 using a Townes Malaise trap placed in Florida scrub habitat in Highlands County. Scrub habitat was located near a "bayhead," a periodically flooded forest dominated by magnolia (Magnoliaceae) and gordonia (Theaceae) trees. Deyrup compared his specimen to specimens identified by

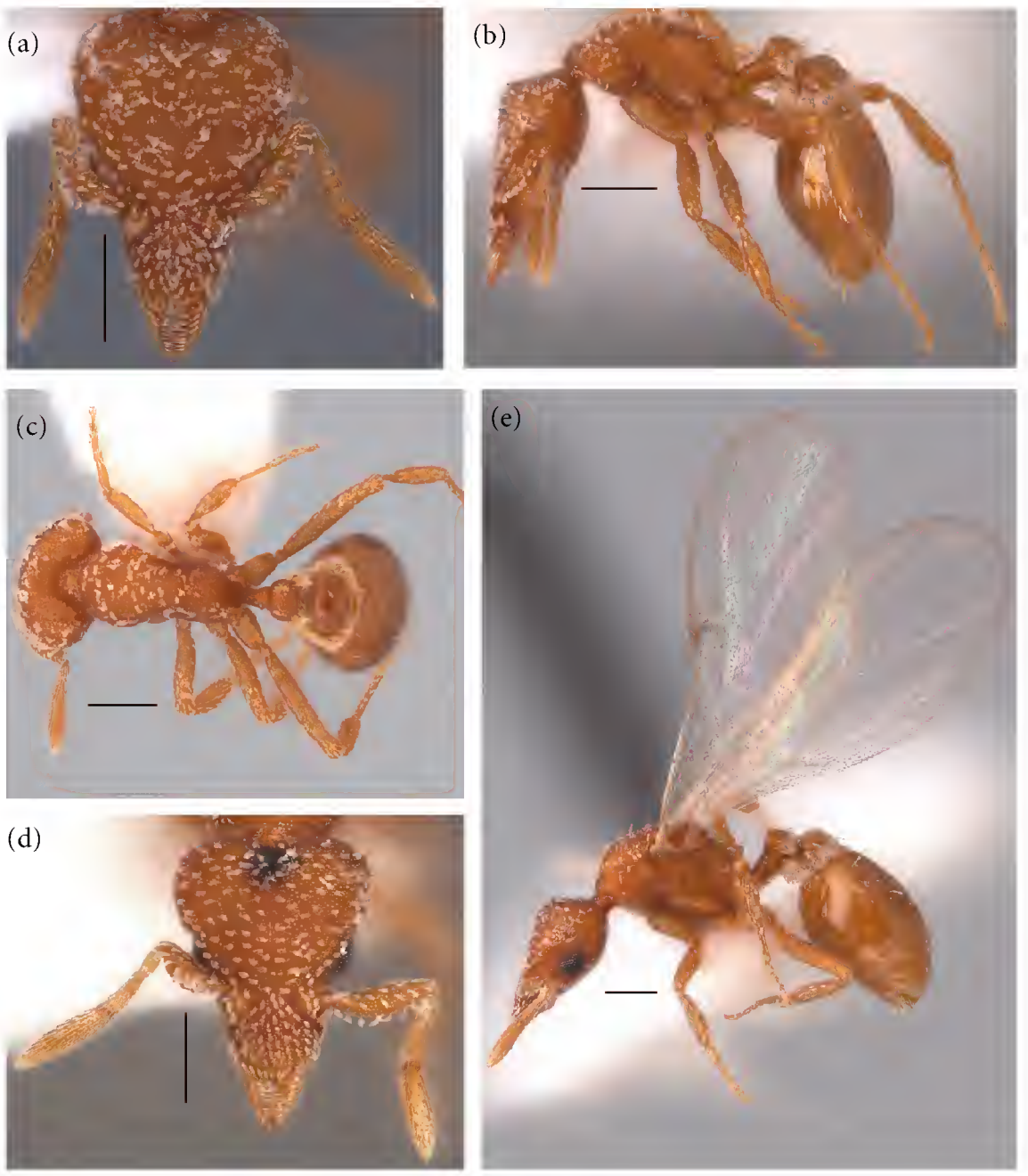

FIGURE 1: *Pyramica epinotalis*: (a) full face view of worker, (b) lateral view of worker, (c) dorsal view of worker, (d) full face view of female, and (e) lateral view of alate female. Scale bar equals 200 μm.

FIGURE 2: Cypress-tupelo swamp north of Gramercy, Ascension Parish, Louisiana.

FIGURE 3: Cypress-tupelo swamp in Jean Lafitte National Historical Park and Preserve, Jefferson Parish, Louisiana.

W. L. Brown, which were collected by R. L. Dressler in Ocosingo, Chiapas, Mexico in 1954 (M. Deyrup, pers. comm.).

Collections of ants in Louisiana were made in cypress-tupelo swamps from spring through fall of 2009 and 2010 on a privately owned tract of land north of Gramercy in Ascension Parish (30°09′48″ N 90°48′39″ W) (Figure 2) and during the late spring and summer of 2011 (May to September) in Jean Lafitte National Historical Park and Preserve in Jefferson Parish (29°47′38″ N 90°06′17″ W) (Figure 3) and Maurepas Swamp Wildlife Management Area (Western Tract) in Saint James Parish (30°06′56″ N 90°40′47″ W)

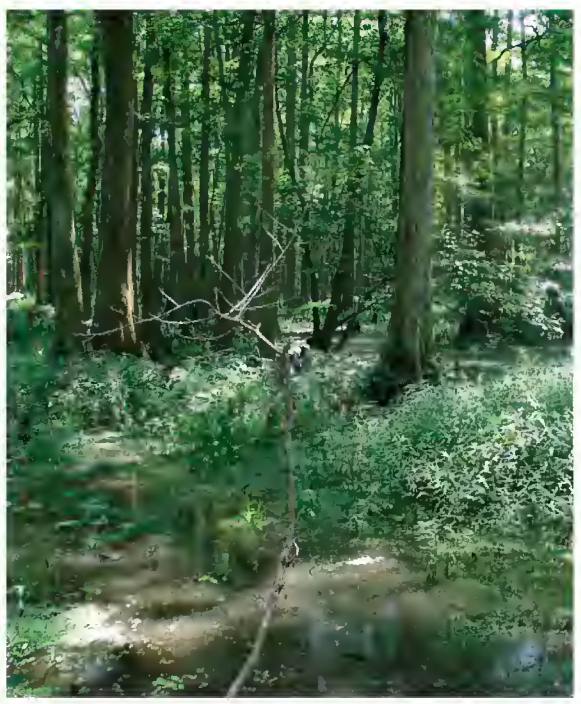

Figure 4: Cypress-tupelo swamp in Maurepas Swamp Wildlife Management Area, Saint James Parish, Louisiana.

Figure 5: Floating pitfall trap shown floating on surface of water in cypress-tupelo-blackgum freshwater swamp.

(Figure 4). All locations are within the Mississippi River deltaic plain in coastal Louisiana.

Cypress-tupelo swamps in Louisiana are characterized and dominated by the presence of bald cypress, *Taxodium distichum* (L.) Rich (Cupressaceae), water tupelo, *Nyssa aquatica* L. (Cornaceae), and red maple, *Acer rubrum* L. var. *drummondii* (Hook. and Arn. Ex Nutt.) Sarg. (Aceraceae) [16]. Collections from floating vegetation were made using a floating pitfall trap in Gramercy, LA, as described by Parys and Johnson [17]. These collections were made as part of a larger study to examine the biodiversity of insects associated with invasive aquatic vegetation. In addition to the characteristic tree species, this site has dense mats of invasive aquatic vegetation formed from common *salvinia* (*Salvinia minima* Baker (Salviniaceae)), water hyacinth (*Eichornia crassipes* (Mart.) Solms (Pontederiaceae)), and water pennywort (*Hydrocotyle* sp. (Araliaceae)). Traps were filled with ethylene glycol as a preservative and emptied at two-week intervals.

Arboreal collections were made on bald cypress (*T. distichum*), water tupelo (*N. aquatica)*, and red maple (*A. rubrum* var. *drummondii)* because they were the most common tree species observed. We chose three to six of each of the aforementioned tree species spaced >50 meters apart for trap deployment. We placed both a cup trap and a bottle trap in each selected tree's canopy and tied a trunk trap at breast height onto the trunk of each tree. Each of the traps was filled with approximately 50 mL ethylene glycol as a specimen preservative.

4.1. Trap Designs

4.1.1. Floating Pitfall Trap (Figure 5). Floating pitfall trap designs were described in detail by Parys and Johnson [17].

4.1.2. Cup Trap (Figures 6(a)–6(e)). We modified a trap that was originally described by Oliveira-Santos et al. [18]. Cup traps were constructed from a single 400 mL tricorner plastic beaker (Figure 6(a)). Three holes were bored into a flange on the rim of the cup (Figure 6(b)). A 6.35 mm cotton rope strand was then threaded through each of the three holes, and hot glue was used to secure each rope (Figure 6(c)). Braided nylon rope was used to link the 6.35 mm cotton rope together at a common point above the trap (Figure 6(d)). The excess cotton rope below the flange on the cup was then taped to the sides of the cup (Figure 6(e)).

4.1.3. Bottle Trap (Figures 7(a)–7(h)). We modified the bottle trap design as described in Kaspari [19]. The traps were created using an inverted 600 mL drink bottle with the base removed (Figure 7(a)). Three holes were bored into the edge of the base (now top) of the container (Figure 7(b)). A foam square (10 cm × 10 cm) was fitted around the base opening of the bottle allowing for at least 2 cm between the opening and the outer edge of the foam square (Figure 7(c)). Fishing line (40 lb test) was tied through the holes in the base of the bottle and around the foam square to connect the square to the bottle (Figure 7(d)). We attached two plastic dowels (6.35 mm dia) to the 10 cm long ends of a length of canvas (40 cm × 10 cm) using hot glue. A hole was burned through the canvas and dowels using a soldering iron; a zip-tie was then attached to the dowel through this hole. A 6 cm hole was also cut into the middle of the canvas 5 cm from one of the dowels. Eight 2 cm slits were then cut 4 cm apart into the canvas around the edge of the hole (Figure 7(e)). The canvas was attached to the base of the bottle using hot glue applied to the inside of the bottle (Figure 7(f)). We tied a 28.3 g fishing weight to the bottle using fishing line (Figure 7(g)). An optional modification to reduce friction on the branches when setting up the traps was to cut the extra tail of canvas to 3 cm width (Figure 7(h)).

4.1.4. Trunk Trap. Trunk trap designs were taken directly from Pinzón and Spence [20]. The only modification was the removal of the plastic flues used to funnel insects into the traps.

4.1.5. Trap Placements. Placement of floating pitfall traps is discussed in Parys and Johnson [17]. The top quarter of

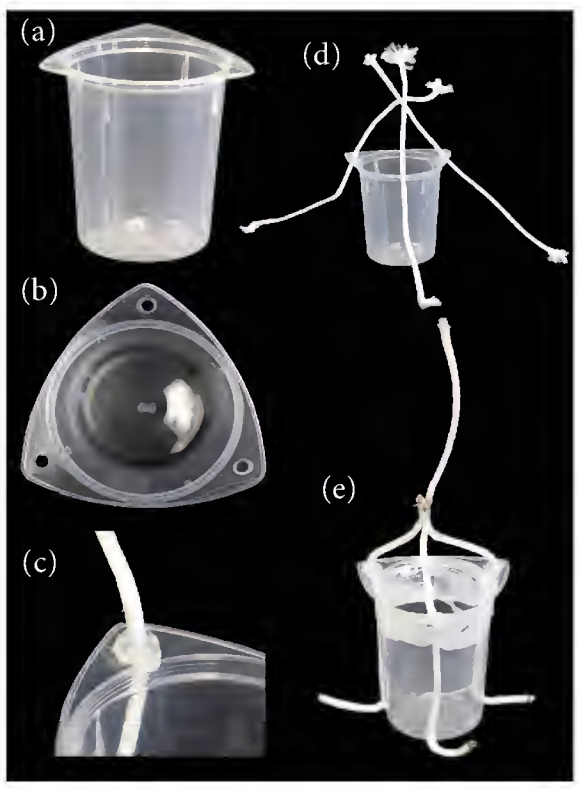

FIGURE 6: Cup trap design: (a) tricorner beaker, (b) holes bored in flange of beaker, (c) cotton rope threaded through flange hole and secured with hot glue, (d) cotton ropes linked together using nylon rope above trap, and (e) completed trap with excess cotton rope below lip of flange taped to sides of beaker (cup).

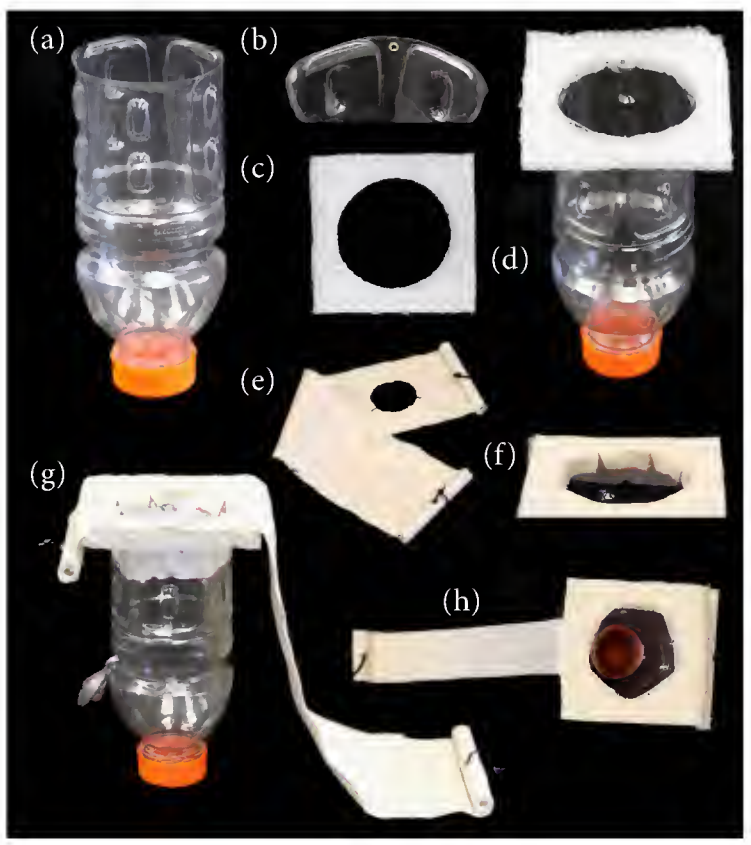

FIGURE 7: Bottle trap design: (a) inverted 600 mL plastic bottle, (b) hole bored into base of bottle, (c) foam square cut to bottle base, (d) foam square place around bottle base with fishing line tied through holes and around square, (e) canvas strip, with hole, slits around hole, dowels at each end, and zip-ties added, (f) canvas attached to inside of bottle with hot glue (g) fishing weight added to bottle with fishing line, and (h) excess canvas trimmed from foam square.

each arboreal trap was brushed with liquid Teflon (Dupont Polymers, Wilmington, Delaware) to prevent ants from escaping. Canopy traps were set in the trees using the same slingshot method as Kaspari [19]. However for ease of sampling and returning the traps to the canopy, the tie-down lines were tied together to make a loop similar to the methods of Oliveira-Santos et al. [18]. For bottle trap designs, the fishing line was connected to the zip-ties attached to the plastic dowels. It is critical that the edge of the cup trap and the edge of the foam on the bottle trap be in contact with a tree branch or trunk to ensure maximum yield of specimens. After each sampling period, the entire contents of the traps were removed, new ethylene glycol was added, and the trap was returned to the canopy.

5. Results and Discussion

Here we report the first records of *P. epinotalis* for the United States. A single alate female was collected by Mark Deyrup in Highlands County, Florida, on 14 August 2009 using a Townes Malaise trap (M. Deyrup, pers. comm.). We collected five females and 14 workers of *P. epinotalis* in Ascension, Jefferson, and Saint James Parishes in Louisiana on various dates from 8 to 21 September 2009 and from 30 May to 23 September 2011. Louisiana collections were made using floating pitfall traps placed directly upon the surface of the water and with cup traps, bottle traps, and trunk traps placed on trunks and branches of three species of trees as described in the methods section. Louisiana collections were made by Katherine Parys, Xuan Chen, and Benjamin Adams. Other collection data are as follows: Florida: Highlands County, Highlands Park Estates, N27.53864, W081.35071, 14 August 2009, M. Deyrup, Townes trap in scrub habitat (near bay-head). Louisiana: Ascension Parish, Gramercy, N of 61 and I-10, 30°09′48″ N 90°48′39″ W, 8–21 Sept; 2009, K. A. Parys, floating pitfall trap in cypress-tupelo freshwater swamp with dense mats of *Salvinia minima* on water surface (1 worker). Jefferson Parish, Jean Lafitte National Historical Park and Preserve, 29°47′38″ N 90°06′17″ W, 30 May–13 June 2011, cup trap on *Nyssa aquatica* branch (1 female); same data except, trunk trap on trunk of *Acer rubrum* var. *drummondii* (1 worker); same data except, 13 June–18 August 2011, bottle trap on *Taxodium distichum* branch (1 female); same data except, trunk trap on trunk of *Taxodium distichum* (2 females); same data except, trunk trap on trunk of *Nyssa aquatica* (2 workers); and same data except, bottle trap on *Acer rubrum* var. *drummondii* branch (1 worker); same data except, cup trap on *Acer rubrum* var. *drummondii* branch (1 female); same data except, 18 August–23 September 2011, bottle trap on *Nyssa aquatica* branch (1 worker); same data except, trunk trap on trunk of *Acer rubrum* var. *drummondii* (2 workers). Saint James Parish, Maurepas Swamp Wildlife Management Area (Western Tract), 30°06′56″ N 90°40′47″ W, 9 June–17 September 2011, trunk trap on trunk of *Nyssa aquatica* (4 workers); same data except, cup trap on *Nyssa aquatica* branch (2 workers).

This is a significant contribution to the distributional record for this species as previously it had only been reported from southern Mexico (Veracruz, Chiapas, Quintana Roo, Tlacocintla), Ecuador (Tiputini), Costa Rica (Limon Province), and Brazil (Mina Gerais) [6, 8, 10]. According to Longino [14], in Costa Rica *P. epinotalis* is known only from the southern Atlantic lowlands, south of Limon. As mentioned in the Natural History section, this

species is thought to be primarily an arboreal species. Similarly, collections in Louisiana were all made from arboreal traps except for a single worker that was collected using a floating pitfall trap, which was in the same habitat type. Xuan Chen also collected ants in leaf litter and quadrats on ground cover in both locations, yet *P. epinotalis* was not found. Although the single Florida collection was made in scrub habitat, the alate female could have flown there from nearby bayhead habitat. Based on the collections made by Longino and other records [8, 13–15], this species may prefer wetland forest habitats. This also appears to be the case with the Louisiana collections, all of which were made in swampy, wetland habitats.

It seems likely that *P. epinotalis* is an introduced species. Evidence for this includes the large geographical gap between the known distribution and the new records of this species from Florida and Louisiana. Other exotic ants (i.e., *Brachymyrmex patagonicus* Mayr, *Pheidole obscurithorax* Naves, *Solenopsis invicta* Buren, and *S. richteri* Forel) from South America have been introduced to the southeastern states [21–24]. However, these species are native to southern South America whereas *P. epinotalis* is more likely native to northern South America, Central America, or southern Mexico making this record more unique. Given the relative novelty of trapping methods used here, it is possible that the range of this species actually may be much more extensive. For example, the range of another introduced arboreal species, *Pseudomyrmex gracilis* (F.), is almost continuous from South and Central America to southern Texas and is now found in Florida, Louisiana, and Mississippi [25, 26]. Dacetine ants native to the USA are not known to nest arboreally but rather have only been reported to nest in rotting wood, soil, or litter [5]. Consequently, most ant collectors in the USA would not consider searching trees for dacetines, given that only a few species, which are primarily tropical, nest arboreally [13].

Typical methods for arboreal collections in USA such as baiting or beating vegetation (unless they are present on outer limbs) would not likely yield dacetines even if they were present. Dacetines are specialized predators [2] and likely would not be attracted to standard baits used to attract other generalist species of ants. Due to their coloration and cryptic habits, these ants could easily be overlooked during visual searches on tree trunks. Furthermore, few ant collectors spend time in wetlands, as most ants in this region are terrestrial. If *P. epinotalis* is truly associated with wetland forests, this species could easily have been overlooked. Collections along the eastern edge of Mexico and into Texas could greatly enhance our knowledge of this species' distribution and provide information on whether or not it is truly an introduced species. Until such time as these collections can be made, we tentatively consider this species to be an exotic species to the US.

Acknowledgments

The authors thank Dr. Julie L. Witbeck with Jean Lafitte National Historical Park and Preserve for facilitating work at that location. They also thank Mr. James Boyce for access to his property in Gramercy as well as Anna Mészáros and Jordan Fryoux for assistance in the field. Thanks to Mark Deyrup for his comments and specimen data, this project was supported by the Northern Gulf Institute (L. Hooper-Bui, Principle Investigator), the Gulf of Mexico Research Initiative (year 1 funds through LSU to L. Hooper-Bui), Mississippi Agricultural and Forestry Experiment Station State Project MIS-311080, the USDA-ARS Areawide Management of Imported Fire Ant Project (Richard L. Brown, Principal Investigator), and Louisiana Department of Wildlife and Fisheries Contracts nos. 673252, 686647, and 696303 (Seth J. Johnson, Principal Investigator). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of this manuscript. This paper was approved for publication by the Louisiana State University Agricultural Experiment Station and approved for publication as journal article number. J-12146 of the Mississippi Agricultural and Forestry Experiment Station, Mississippi State University.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 764350, 7 pages
doi:10.1155/2012/764350

Research Article

Mother-Offspring Relations: Prey Quality and Maternal Size Affect Egg Size of an Acariphagous Lady Beetle in Culture

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Received 10 January 2012; Revised 21 March 2012; Accepted 9 April 2012

Academic Editor: Michael Rust

We investigated mother-offspring relations in a lady beetle *Stethorus punctillum* Weise that utilizes spider mites as prey. Our objectives were to determine if (1) prey quality affects egg size, (2) maternal size correlates with egg size, and (3) egg size affects hatching success. We fed predators spider mites *Tetranychus urticae* Koch from lima bean *Phaseolus lunatus* L. foliage in the laboratory. Mothers of unknown body size offered high rather than low quality spider mites since birth produced larger eggs. Mothers of known body size offered only high quality spider mites, produced eggs of variable size, but mean egg size correlated positively with hind femur length. Mothers laid their eggs singly, rather than in batches, and eggs were large relative to femur size. Egg size did not affect hatch success; mean hatch rate exceeded 95% regardless of egg size. In conclusion, the quality of prey consumed by *S. punctillum* mothers while in the larval stage can affect their size as adults and, consequently, the size of their eggs. The behavior of laying eggs singly, the positive relationship between maternal size and mean egg size, and the high rate of egg hatch suggest that *S. punctillum* mothers invest heavily in offspring.

1. Introduction

Maternal investment in offspring is a major topic that has intrigued zoologists for years [1]. Trade-offs between allocating limited resources to offspring rather than to extending the lifespan of the mother exist [2]. Researchers commonly use offspring (egg) size as an estimate of the amount of resources allocated to offspring [3]. In fact, life history theory predicts that there is a trade-off between egg number, that is, potential or realized fecundity, and egg size in a diversity of animals [4, 5].

One prominent factor that could affect egg size of a predator is the quality of prey available to the mother. Mothers that consume high quality prey should maximize the amount of resources allocated to their offspring [2]; however, this is not always the case [6]. Presumably, mothers fed high quality prey should transfer essential nutrients into egg production rather than to general maintenance and survival. A number of studies attest to this assumption. Although evidence that prey quality affects egg size of predatory insects is not robust, the staphylinid beetle *Tachyporus hypnorum* F. produced smaller rather than larger eggs when fed aphids of low quality rather than fruit flies of high quality [7]. Carabid beetles (*Pterostichus melanarius* Illiger, *Pterostichus cupreus* L.) produced larger eggs when fed a mixed diet of carbohydrates and protein rather than a diet of aphids alone [8]; apparently, a mixed diet was more nutritious for the species tested by Wallin et al. [8]. These two exemplary studies tested insect predators that lay eggs singly rather than in batches. There are no published records on the effect of prey quality on egg size of coccinellids that lay eggs singly rather than in batches. For coccinellids that lay their eggs in batches, consumption of low quality prey can result in a reduction in batch size [9]. Food stress (scarcity of aphids) can lead to a reduction in the size of egg batches, but not individual eggs within batches in the coccinellid *Coccinella septempunctata* L. [10].

Maternal body size may correlate with the size of progeny in animals. At an interspecific level, there is evidence that large-sized species produce small eggs, whereas small sized species produce large eggs relative to their body size [5]. This mother-offspring size relationship occurs between

coccinellid species. For example, large-sized coccinellids, such as *Adalia* species and *Coccinella* species, produce small eggs relative to their body size, whereas tiny-sized *Stethorus* species produce large eggs relative to their body size [9, 11]. Differences in egg size between small and large species might be an adaptation to the size of their prey and the size of prey aggregations on host plants [11–13]. It may also reflect the oviposition strategies of a species (i.e., degree of synovigeny, number of ovarioles present in a species, and rate of egg maturation). Note that egg-laying differences between species with distinct life histories could play a role in egg size variation. For example, acariphagous coccinellids such as *Stethorus punctillum* Weise and *Stethorus punctum punctum* (LeConte) lay their eggs singly rather than in batches [14]. In contrast, large-sized aphidophagous species such as *Adalia bipunctata* L. and *C. septempunctata* L. lay eggs in batches [15].

At the intraspecific level, body size of females of a given species can scale positively, negatively, or not at all with egg size [16–19]. When Kajita and Evans [20] combined data on five aphidophagous coccinellids, mean egg size (volume) related significantly to maternal body weight, with no significant relationship among species. Egg size does not correlate with body size in the carabid beetle *Brachinus lateralis* Dejean, an ecto-parasitoid of aquatic beetles [21], or in the colydiid beetle *Dastarcus helophoroides* Faimaire, an ecto-parasitoid of cerambycid beetles [22].

Intuitively, if large-sized eggs contain more essential nutrients than intermediate or small-sized ones within a species, the larger eggs should have the greatest fitness. However, egg contents do not always track along with egg size [23], and the amount of yolk provisioned within an egg could provide a more accurate estimate of maternal investment than egg size [1]. McIntyre and Gooding [23] showed that egg size correlated negatively or not at all to hatch rate in house flies. Egg size does not influence hatch time of *C. septempunctata* [10]. There are no other records on the effects of egg size on hatch rate in coccinellids. First instar larvae of two carabids, *P. cupreus* and *P. melanarius*, hatching from large rather than small eggs had longer survival rates [8]. In the staphylinid beetle *Aleochara bilineata* (Gyll.), larger first instar larvae survived longer and were more efficient at finding hosts than smaller-sized ones [24].

In this study, we examined the relations between maternal size and egg size in *S. punctillum*, a tiny lady beetle that utilizes spider mites as essential prey [14, 25, 26]. Our research objectives were to determine if (1) prey quality affects egg size, (2) maternal size correlates with egg size, and (3) egg size affects hatching success. This information will expand our knowledge of the life history strategies of predatory insects, especially coccinellids.

2. Materials and Methods

2.1. Plants and Arthropods. Lima bean *Phaseolus lunatus* L. were grown from seed in plastic planters (57 × 35.5 × 16 cm, L × W × H) in an environmentally controlled greenhouse (at 27°C, 30–40% RH, and 18 hr photophase) in Stoneville, Mississippi, USA. In each planter was approximately 4 g of fertilizer (Osmocote, Smart-Release, The Scotts Company LLC) with 2.5 L of potting soil (Miracle-Gro, Moisture-Control, The Scotts Company LLC), 2.5 L of coarse vermiculite, and water before adding seeds. We obtained two-spotted spider mite *Tetranychus urticae* Koch adults from Syngenta Bioline (Oxnard, California, USA) and predator *S. punctillum* adults from Applied Bionomics Ltd. (Sidney, B.C. Canada). Male and female predators were placed together in clear plastic containers (16 × 12 × 6 cm, L × W × H, with screened lids). Their progeny was separated, according to stage of development (egg, larval, pupal, and adult) and reared in Petri dish arenas (90 × 25 mm, W × H, with screened lids) in a growth chamber (23°C, 60% RH, 16 h photophase).

2.2. Prey Quality and Egg Size. To determine the effect of prey quality on egg size, we reared predators from the time of egg eclosion on spider mites from one of two cultivars, Henderson Bush or Fordhook 242 lima bean. Spider mites ingest a much higher dose of linamarin when feeding on foliage of the Henderson cultivar than the Fordhook cultivar, and consumption of Henderson-fed prey causes a reduction in growth, development, and body size of *S. punctillum* larvae [27]. Therefore, we designated low quality and high quality prey as spider mites from the Henderson and Fordhook cultivar, respectively. Predator larvae were reared in Petri dish arenas (90 × 25 mm, W × H, with screened lids), at a density of 20 first instars. Arenas were stored inside a growth chamber at 23°C, 60% RH, and 16 h photophase. Immature predators remained in the same arenas until adult emergence. Newly emerged females were confined with males in clear plastic containers (16 × 12 × 6 cm, L × W × H, with screened lids) on mite-infested Henderson or Fordhook leaves (the same treatment used for immature stages) for 4 days, to allow mating. A small ball of cotton, moistened with distilled water, was in each arena for beetles. Containers were stored inside a growth chamber under the same conditions of temperature, humidity, and photophase as previously described. After this, we placed the females, now presumed to have mated, individually in Petri dish arenas provisioned with 1-2 mite-infested leaves. We ensured that leaves harbored enough mites to meet the daily nutritional requirements of mothers and replaced old leaves with new leaves each day. We conducted one trial of this experiment with sample size of 10 mothers in the low quality and 10 mothers in the high quality prey treatments. We consistently used young mothers of approximately the same age in the two treatments and examined eggs laid by them over three consecutive days (19–21 July 2010), near the beginning of their oviposition period. Each day we harvested single eggs using a fine, camel hair paintbrush. The laying of eggs singly, rather than in batches, is typical for most, if not all, species in the genus *Stethorus* [14]. We transferred eggs to clean Petri dishes. They laid from 2 to 9 eggs per day (132 total eggs) in the low quality and 4 to 9 eggs per day (182 total eggs) in the high quality treatments. Each day, we measured the size (length, width) of freshly laid eggs of each

female using an Olympus SZ11 zoom stereomicroscope and computer-based imaging software (Image Pro Plus, Media Cybernetics Inc., MO, USA). We also determined the effects of date of collection of eggs (harvest date) and prey quality on egg length. We calculated mean egg volume (V) using the formula for a prolate spheroid, $V = (4/3)\pi a^2 b$, where a represents half the mean egg width and b represents half the mean egg length, after Kajita and Evans [20]. We did not measure body size of mothers in this experiment.

2.3. Maternal Size and Egg Size. In a separate experiment to determine if egg size related to maternal size, we reared *S. punctillum* mothers from the time of eclosion in Petri dish arenas (90 × 25 mm, W × H, with screened lids), at a density of 20 first instars per arena, on spider mites from Fordhook lima bean only. Arenas were stored inside a growth chamber (23°C, 60% RH, 16 h photophase) and immature predators remained in the same arenas until adult emergence. Newly emerged females were confined with males in clear plastic containers (16 × 12 × 6 cm, L × W × H, with screened lids) on mite-infested Fordhook leaves for 4 days, to allow mating. Procedures of maintaining predators were the same as those detailed in the previous experiment. In this experiment, egg size and maternal size were determined using a stereomicroscope and imaging software (as before). Based on allometry [28], we assumed that the hind femur was a good estimate of the body size of *S. punctillum* mothers. Therefore, we correlated the length of the mother's hind femur to the length, volume, as well as perimeter of her eggs. In order to lighten the workload, we conducted two identical trials with 18 mothers in trial 1 and 22 mothers in trial 2. We consistently used young mothers of approximately the same age in the two trials and examined eggs laid by them, from near the beginning of the oviposition sequence until they ceased producing eggs or died. We harvested eggs from arenas from late February to early April 2010 in trial 1 and from early May to early July 2011 in trial 2. The total number of eggs laid per mother over the trial 1 test period ranged from 11 to 176 eggs (69 eggs, average) and an average of 15 harvest dates per mother, yielding an average of 4 eggs per date. The total production from 18 mothers was 1,218 eggs in trial 1. The total number of eggs laid per mother during trial 2 ranged from 9 to 128 eggs (48 eggs, average) and an average of 11 harvest dates per mother, yielding an average of 4 eggs per date. The total production from 22 mothers was 1,061 eggs in trial 2. We transferred eggs to clean Petri dishes, measured the size of freshly laid eggs using a stereomicroscope and imaging software (as before). We did not determine the effects of harvest date on egg length. We did calculate egg volume from mean egg length and egg width (as before).

2.4. Egg Size and Hatching. To determine if egg size influenced hatching, we monitored the days required to hatch and the percent hatch of eggs laid by the 22 mothers in trial 2 of the previous experiment. We placed eggs laid by each mother each day into a clean Petri dish (40 × 10 mm, W × H, with unscreened lids) and checked each dish daily for egg hatch, and promptly removed any hatching first instars. For each mother, we compared the number eggs that hatched with those that did not hatch and correlated hatch time (days) and percent hatch rate with egg length. As an additional analysis, we assigned eggs into size categories (short, 341–360 μm; intermediate, 361–380 μm; long, 381–400 μm) to determine if size affected hatch rate or hatch time. The sample size was 22 mothers (1,032 hatched eggs; 29 unhatched eggs).

2.5. Statistical Analysis. We used the Student's t-test to determine the effect of prey quality on egg length and volume and a two-factor analysis of variance (2-way ANOVA) to determine the effect of harvest date and prey quality, with interactions, on egg length and the Holm-Sidak method to separate means, if necessary. We used a Pearson Product Moment Correlation to determine if maternal size correlated with egg size, and a Simple Linear Regression to estimate a functional relationship between femur length and egg size. We also used the Student's t-test to compare the length of hatched versus unhatched eggs and a Pearson Product Moment Correlation to determine if hatch time or hatch rate correlated with mean egg length. We used a one-factor analysis of variance (1-way ANOVA) to detect any differences in hatch time or hatch rate between egg categories (short, intermediate, or long) and the Holm-Sidak method to separate means, if necessary. We analyzed data following a randomized design, and square root transformed absolute data and arcsine transformed percentage data prior to analysis [29]. Mean values were considered significantly different when $P \leq 0.05$. We used Sigma Stat 3.0.1 (Systat Software Inc., Richmond, CA, USA) software for analysis of data. All data presented herein represent nontransformed values.

3. Results

In our first experiment, egg length and volume were greater for mothers feeding on high quality rather than low quality prey for pooled data (egg length, $t = 5.0$; df = 18; $P < 0.001$; Figure 1(a); egg volume, $t = 8.0$; df = 18; $P < 0.001$; Figure 1(b)). The date that eggs were harvested from mothers did not affect egg length ($F = 0.90$; df = 2, 308; $P = 0.41$). The mean ± SEM egg lengths on consecutive dates were 391.5 ± 3.6 μm (day 1), 384.8 ± 2.7 μm (day 2), and 381.6 ± 3.0 μm (day 3) in the high quality treatment and 370.4 ± 3.2 μm (day 1), 369.2 ± 2.8 μm (day 2), and 373.2 ± 3.4 μm (day 3) in the low quality treatment. Longer eggs resulted from mothers fed high quality rather than low quality prey ($F = 31.9$; df = 1, 308; $P < 0.001$) with no interaction between harvest date and prey quality ($F = 1.8$; df = 2, 308; $P = 0.17$).

In our second experiment, hind femur length correlated with mean length, volume, and perimeter of eggs (Table 1). Mean egg size increased as femur length increased (Figures 2(a) and 2(b)). Note that egg size is large relative to femur size in this species (Figures 3(a) and 3(b)). The size of eggs laid by mothers was variable, but significant changes in egg size within and between harvest dates were not apparent.

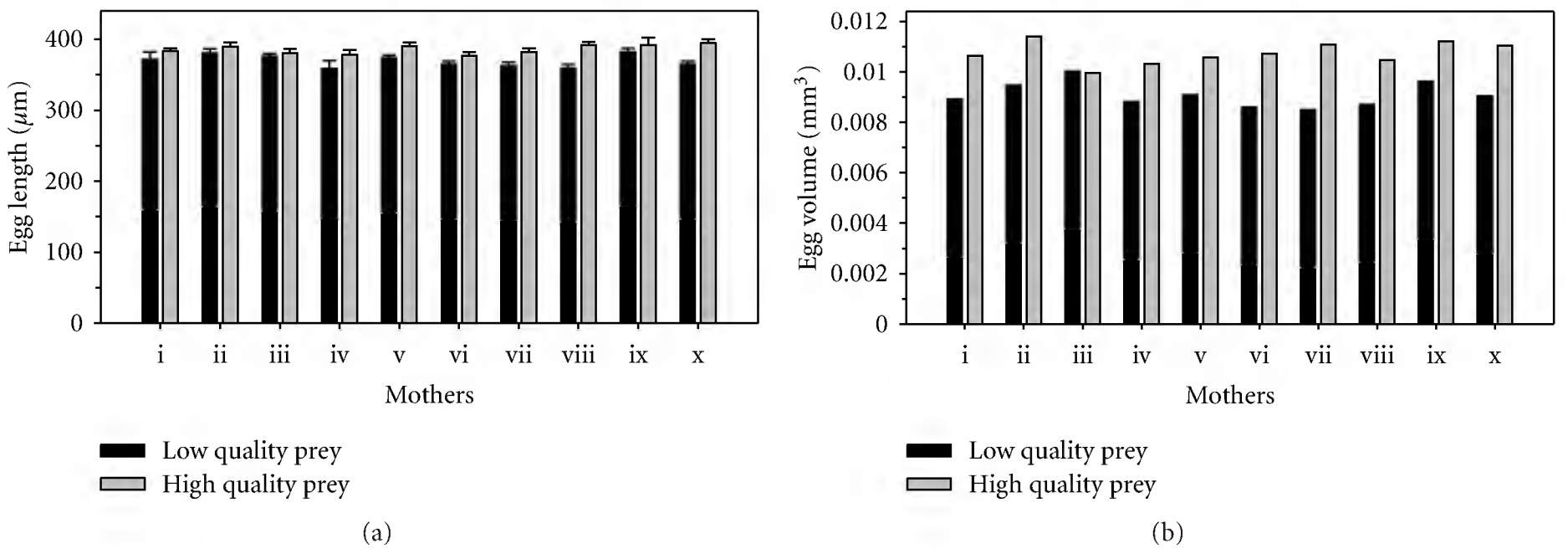

Figure 1: Mean ± SEM length (a) and volume (b) of eggs laid by 10 predators (mothers) fed high quality or low quality prey over combined harvest dates. Egg volume generated from mean egg length and mean egg width. High quality or low quality prey represent spider mites that fed on foliage of Fordhook or Henderson lima bean, respectively.

Table 1: Linear regression equations and ANOVA statistics for the relationship of egg size versus femur length of predators (mothers).

Trial	Egg size	Equation	F	df	P	r	r^2
1	Length	$Y = 162.6 + 0.6X$	6.0	1, 16	0.03	0.52	0.27
	Volume	$Y = -0.02 + 0.007X$	9.0	1, 16	0.008	0.60	0.36
	Perimeter	$Y = 517.4 + 1.3X$	10.4	1, 16	0.005	0.63	0.39
2	Length	$Y = 110.7 + 0.7X$	25.6	1, 20	< 0.001	0.75	0.56
	Volume	$Y = 0.003 + 0.00002X$	4.6	1, 20	0.045	0.43	0.19
	Perimeter	$Y = 468.8 + 1.4X$	41.4	1, 20	< 0.001	0.82	0.67

Simple linear regression, $Y = b_o + b_1X$; Y represents egg length, volume or perimeter; b_o and b_1 represent the constant term and slope, respectively; X represents femur length. Length and perimeter are in μm units. Volume is in mm^3 units. Regression equations reflect nontransformed data. See scatterplot (Figure 2) with regression lines of egg length versus femur length. Regression lines of egg perimeter versus femur length and egg volume versus femur length not plotted.

In our final experiment, the length of eggs did not correlate with the time required for first instar larvae to hatch ($r = 0.03$; $P = 0.8$; $n = 22$; Figure 4(a)) or with percent hatch rate ($r = 0.19$; $P = 0.4$; $n = 22$; Figure 4(b)). Assignment of eggs to categories (short, intermediate, long) did not reveal any significant effect of size on hatch time ($F = 0.9$; df = 2, 19; $P = 0.4$) or hatch rate ($F = 0.6$; df = 2, 19; $P = 0.5$) in this study (Table 2). First instar larvae usually hatched within 5 to 6 days after the day of oviposition. Hatch rate was relatively high, ranging from 89% to 100%. There was no difference in the length of eggs that hatched versus did not hatch ($t = 1.1$; df = 34; $P = 0.26$). The mean ± SEM length of hatched and unhatched eggs was 370.4 ± 2.3 μm and 375.4 ± 3.9 μm, respectively. Just 14 out of 22 mothers laid eggs that failed to hatch.

4. Discussion

Our observation that *S. punctillum* mothers produce larger eggs when fed from birth on high quality prey (spider mites on Fordhook lima bean) suggests that the nutritional content of prey has important consequences on the growth of this predator. In our prey quality experiment, we did not determine the body size of mothers. At the time, we did not think it was necessary to record their size, since our previous research clearly showed that prey quality altered the growth and development of *S. punctillum* immatures; smaller immatures resulted from feeding on low-quality rather than high-quality prey [27]. The concentration of linamarin, a cyanogenic glycoside purportedly used by the plant in defending itself against herbivory, is much greater in Henderson than in Fordhook lima beans [30]. Linamarin reduces the amount of nutrients (including soluble protein) that *T. urticae* can extract from lima bean foliage, resulting in prey of lower quality for *S. punctillum*, reducing growth rate and body size, but not fecundity or longevity [27]. Molecules that plants use in defense against herbivory might pass up the food chain [31] and reduce the quality of prey for the predator [32] or have no negative effects on the predator [33]. Prey quality can affect egg size in other natural enemies. Carabid beetles that consume high quality prey produce larger eggs than those that consume low quality prey [8]. Staphylinid beetles that consume aphids of high quality produce larger eggs [7]. No prior study demonstrates that prey quality affects the size of individual eggs of coccinellids. One study does demonstrate that low quality prey reduces the size of egg batches of coccinellids [9].

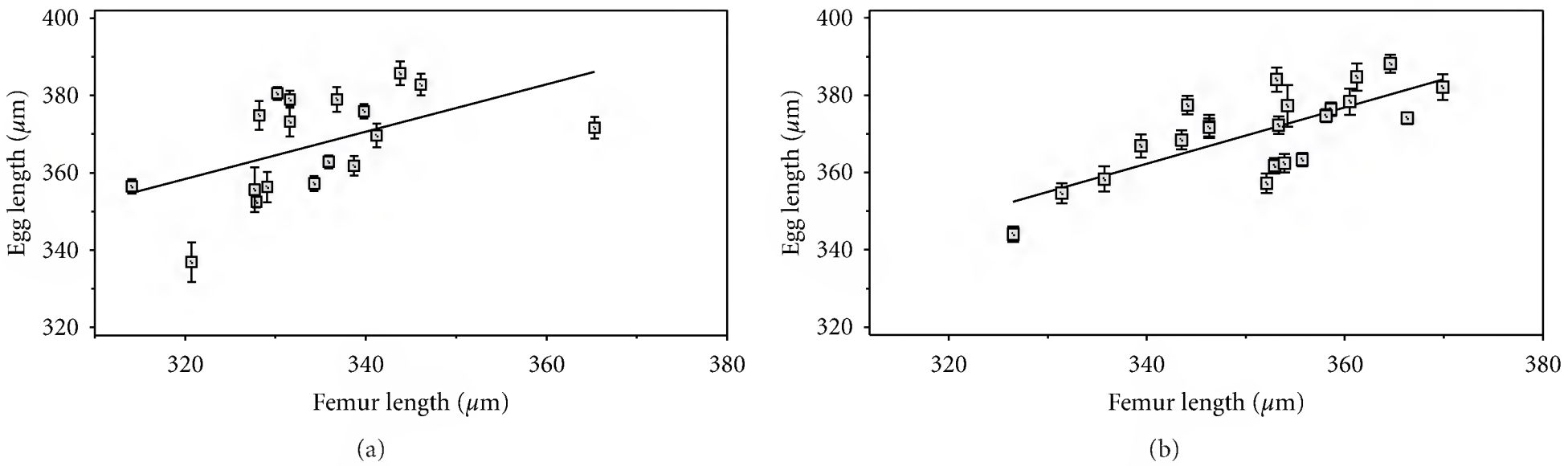

FIGURE 2: Mean ± SEM egg length versus femur length of combined mothers fed only high quality prey in trials 1 (a) and 2 (b) over combined harvest dates. The regression line defines a significant relationship between mean egg length (Y) versus femur length (X), simple linear regression $Y = b_o + b_1X$. In trial 1: b_o, 162.6; b_1, 0.6; $r^2 = 0.27$; n, 18 observations (mothers). In trial 2: b_o, 114.6; b_1, 0.7; $r^2 = 0.55$; n, 22 observations (mothers). Refer to Table 1 for the ANOVA statistics of each regression analysis.

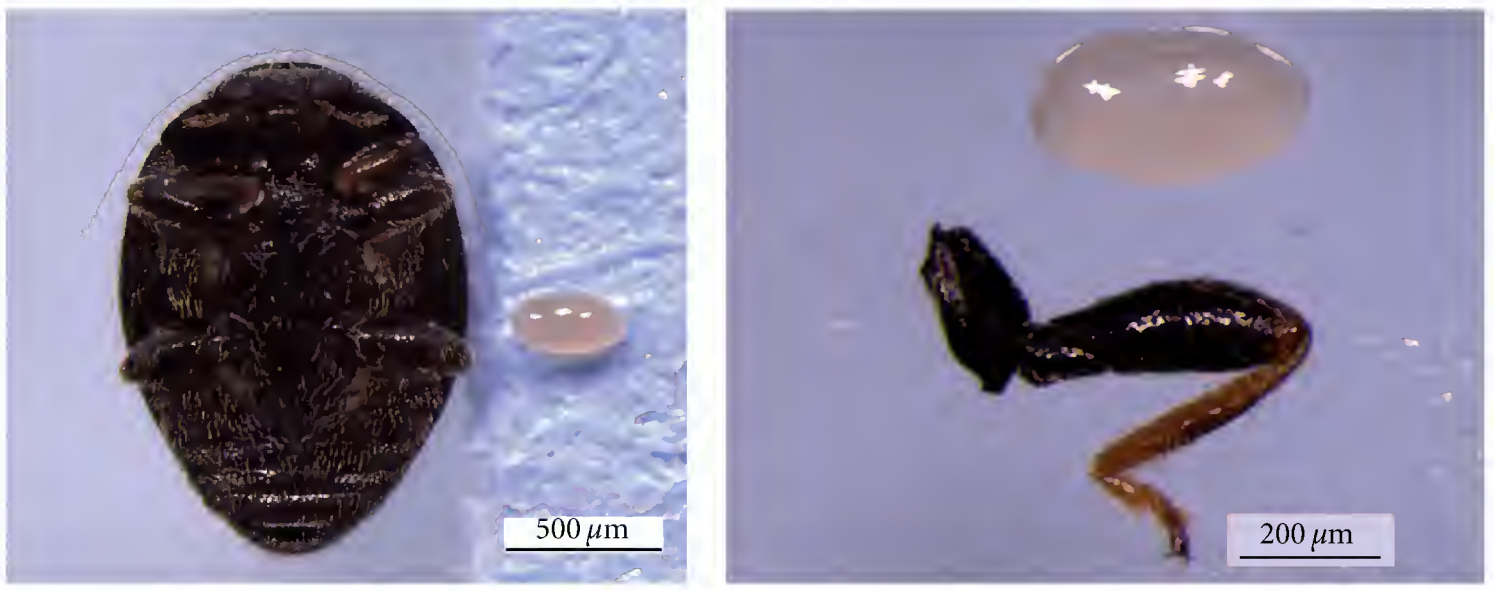

FIGURE 3: Ventral view of a mother with freshly laid egg (a) and dissected femur of another mother adjacent to one of her eggs (b).

Our observations that *S. punctillum* body size correlates with mean egg size (even when predators are reared on the same high quality prey) may suggest that the quantity of prey nutrients that developing larvae ingest and metabolize is fine-tuned with the size they attain as adults and the average size of their progeny (eggs). To our knowledge, no one has examined the relationship between body size and egg size in *S. punctillum*, or any other acariphagous lady beetle, so a mechanism that explains this positive maternal-offspring relationship in this lady beetle is unavailable. At the intraspecific level, body size of females of a given species can scale positively, negatively, or not at all with egg size [16–19]. When Kajita and Evans [20] combined data on five aphidophagous coccinellids, mean egg size (volume) related significantly to maternal body weight, with no significant relationship among species. Investigators report a lack of correlation between body size and egg size in carabid tiger beetles [21], colydiid beetles [22], and spiders [19].

Stethorus punctillum mothers are sensitive to changes in the quantity of prey (tetranychid mites) available to them. Any significant decrease in food supply available to ovipositing mothers can halt subsequent egg laying behavior and oogenesis [EWR & ZW, unpublished data], [34]. Therefore, the constant availability of nutritious prey is critical to the reproductive success of this specialized predator.

The small size of *S. punctillum* adults may place constraints on (1) the number of ovarioles within their ovaries, (2) the rate of oogenesis, (3) the capacity to store many eggs, and (4) the rate at which they lay eggs. *S. punctillum* emerge as adults with no developing eggs in their ovaries, and all adult females are limited to only two ovarioles per ovary [EWR, unpublished data], [35]. Consequently, mothers might invest more resources (nutrients) into producing fewer large eggs rather than many small eggs to compensate for these limitations.

Our observation that egg size does not influence hatch time or hatch rate is encouraging and suggests that egg size is not a good predictor of egg viability. Egg size also does not influence hatch time of the seven spot ladybird beetle *C. septempunctata* L. [10]. The moderately high hatch rates evidenced in this study are the expectation for species, such as *Stethorus* spp., which invest considerable resources into producing fewer, larger eggs. The fact that *S. punctillum* mothers lay eggs singly and do not experience high rates of

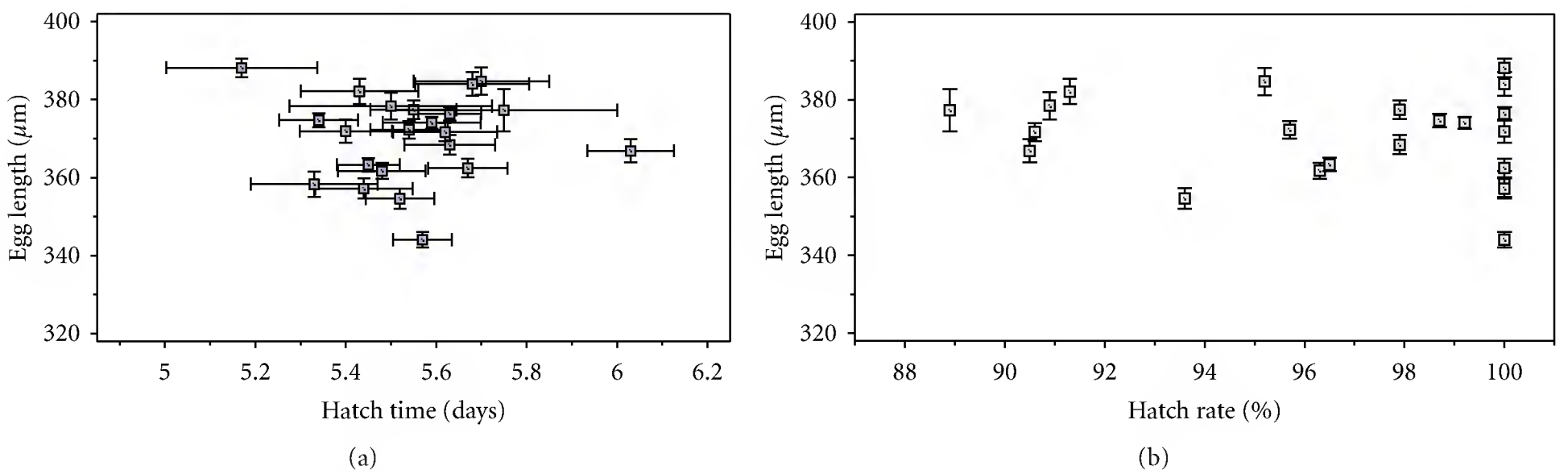

FIGURE 4: Mean ± SEM egg length versus mean ± SEM hatch time (a) or hatch rate (b) of eggs from 22 mothers, from trial 2 of the maternal size-egg size experiment. Mothers fed high quality prey only.

TABLE 2: Mean ± SEM hatch rate and hatch time of predator eggs in relation to size category.

Egg length range (μm)	Category	Hatch rate (%)	Hatch time (days)	Mothers (n)
341–360	Short	98.4 ± 1.60	5.46 ± 0.05	4
361–380	Intermediate	95.9 ± 1.07	5.58 ± 0.05	14
381–400	Long	96.6 ± 2.10	5.49 ± 0.12	4

Only hatched eggs represented in this table.

cannibalism fits an oviposition strategy of investing heavily into eggs. We did not determine if *S. punctillum* larvae hatching from short rather than intermediate or long eggs were less likely to reach the pupal stage in this study. Others have shown that larvae of two predatory carabids, *P. cupreus* and *P. melanarius*, which hatch from large rather than small eggs, lived longer [8]. Larger-sized first instar larvae of a staphylinid *A. bilineata* survive longer and are more efficient at finding hosts than smaller-sized ones [24].

In conclusion, *S. punctillum* mothers that consume high-quality prey during the larval and adult stages generate larger eggs. Maternal body size affects progeny size; egg size increases as femur length increases. Longer eggs do not develop more rapidly or hatch at higher rates than shorter eggs or vice versa. Further research could explore the influence of feeding rate and oogenesis on the size relationship between *S. punctillum* mothers and their progeny. Since our knowledge of body size-egg size relationships is limited to only several coccinellid species, further investigations on the subtle effects of nutrition on predator size are warranted. Only then can we identify patterns that would allow us to predict the degree that coccinellid mothers will invest resources (nutrients) into eggs rather than into sustaining their lifespan.

Acknowledgments

The authors thank John J. Sloggett and Jean-Louis Hemptinne for insightful reviews of an earlier version of this paper. They thank M. Guadalupe Rojas for bean foliage infested with spider mites. Anonymous reviewers gave recommendations that improved this paper. The United States Government has the right to retain a nonexclusive, royalty-free license in and to any copyright of this paper. This paper reports the results of research only. Mention of a commercial or proprietary product does not constitute an endorsement of the product by the United States Department of Agriculture. USDA is an equal opportunity provider and employer.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 275128, 6 pages
doi:10.1155/2012/275128

Research Article

Volatile Chemicals of Adults and Nymphs of the *Eucalyptus* Pest, *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae)

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Received 23 February 2012; Accepted 21 April 2012

Academic Editor: Jocelyn G. Millar

Thaumastocoris peregrinus is an introduced "true bug" that is now a severe pest in *Eucalyptus* plantations of various Southern Hemisphere countries. The semiochemicals of thaumastocorids are completely unknown. Therefore, volatile chemicals from *T. peregrinus* nymphs and adults were identified as possible leads for pheromones potentially useful for control. The contents of nymphal exocrine glands, which are shed at molting, were identified from extracts of exuviae. Adults lack functional metathoracic scent glands that are characteristic of most heteropterans; however, both males and females possess a glandular-appearing hold-fast organ that they quickly extrude posteriorly when disturbed. Whole body hexane extracts from males and females were prepared by freezing the insects in a flask so that they extruded the hold-fast organ, and then they were extracted with hexane. Volatiles from nymphal exuviae included benzaldehyde, octanol, (*E*)-2-octenol, octanoic acid, decanal, and hexanoic acid. Adult volatiles included 3-methylbut-2-en-1-yl butyrate and 3-methylbut-3-en-1-yl butyrate.

1. Introduction

Thaumastocoris peregrinus Carpintero and Dellapé (Heteroptera: Thaumastocoridae) is an introduced pest of nonnative *Eucalyptus* plantations in various countries in Southern Hemisphere (e.g., South Africa, Argentina, Uruguay, and Brazil) [1–3]. In 2005, it was first found in Buenos Aires, Argentina, on *Eucalyptus viminalis*, *E. tereticornis*, and *E. camaldulensis* [1]. In Brazil, *T. peregrinus* was first found in 2008, on a hybrid clone of *E. grandis x E. urophylla* in São Francisco de Assis, Rio Grande do Sul, and on *E. camaldulensis* trees in Jaguariaúna, São Paulo [4]. Initial studies on life history of *T. peregrinus* were done in Australia [5]; however, no investigation was performed on semiochemicals from these insects.

Heteropteran nymphs and adults characteristically produce allomones for defense; typically, the defensive secretions of nymphs are produced in dorsal abdominal glands (DAGs) [6]. The contents of DAGs are shed along with the exuviae each time the nymph molts, and extraction of exuviae is a convenient method to obtain the DAG secretion [7]. Adult heteropterans characteristically possess metathoracic scent glands from which they release irritating secretions [6]. However, examination of *T. peregrinus* adults by one of us (JRA) revealed that the metathoracic glands are vestigial (unpublished data). On the other hand, adults and nymphs of these unusual bugs possess a rectal organ, similar to that described for plant bugs (Miridae) [8] that is everted when the insects are disturbed. The *Thaumastocoris* rectal organ has a glandular appearance and instantly sticks the insects to the substrate when the insects are disturbed and can as quickly be released (JRA, personal observation) (Figure 1). Pheromones are known for members of several heteropteran families [9], but the semiochemicals of *T. peregrinus* and

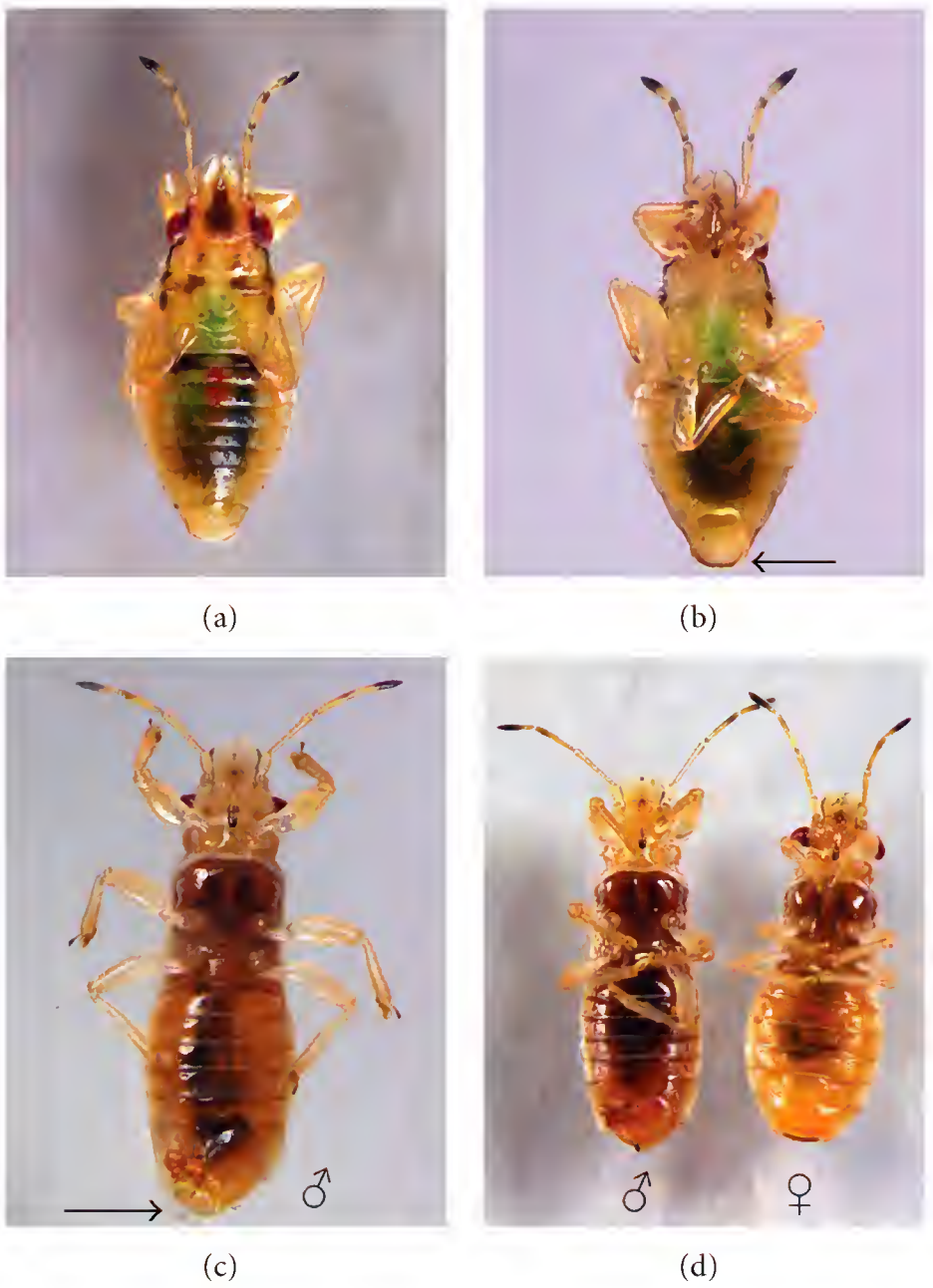

FIGURE 1: *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae) male, female, and nymph. (a) Dorsal view of a 5th-instar nymph; (b) nymphal ventral view showing the rectal organ (arrow); (c) ventral view of the male showing the everted rectal organ (arrow); (d) male and female ventral view with the rectal organ not exposed.

other thaumastocorids are completely unknown. Therefore, the volatile chemical compounds present in the exuviae for the five nymphal instars, and both adult sexes were identified and quantified.

2. Materials and Methods

2.1. Insect Rearing and Extractions. Insects were obtained from a colony at EMBRAPA Florestas, Colombo, Paraná, maintained in the Laboratório de Semioquímicos, Departamento de Química of the Universidade Federal do Paraná (UFPR) under controlled laboratory conditions of 25°C ± 2 and 12 L: 12 D. Adults and nymphs were reared on branches of *Eucalyptus benthamii* in acrylic boxes (30 cm × 30 cm × 30 cm) until their use. To obtain exuviae and virgin adults for the extractions, nymphs of each instar were held individually in small round plastic containers (2.5 cm of diameter) with a gel (Hydroplan—EB/HyC, SNF S.A. Floger) in the bottom of the container for moisture and a leaf disc of *E. benthamii* on the gel. Leaf discs were changed every other day. After nymphs molted, exuviae were collected for extraction, and recently emerged males and females were isolated. Males or females of the same emergence date were grouped in Petri dishes (5 cm of diameter) containing gel and a leaf disc until the extraction. Fifth instar nymphs were grouped in cages provisioned as above to obtain mated males and females for extraction. Couples were formed within 2 days of emergence, and extractions of adults were performed only after eggs were present, which confirmed the mated status of adults.

2.2. Extraction of T. peregrinus Exuviae (1st–5th Instar). Exuviae were extracted with 180 μL of hexane for 24 hours. Each extraction was made with the exuviae available in that day, with a minimum of 12 and maximum of 24 exuviae. At least three repetitions were made for each instar, consisting of at least 45 exuviae in total. After extraction, tridecane (ca. 10 ppm) was added to each sample as an internal standard (IS); the final concentration of the IS was calculated for each extract. Extracts were concentrated and analyzed using a gas chromatograph (GC-2010—Shimadzu) and a gas chromatograph coupled with a mass spectrometer (GC-MS-QP 2010 Plus—Shimadzu). The detected compounds were quantified based on the area of the IS. The GC was equipped with a RTX-5 column (30 m × 0.25 mm i.d. and 0.25 mm film thickness; Restek, Bellefonte, PA, USA). One μL of extract was injected into the GC using the splitless mode with injector temperature at 250°C. The column oven temperature was maintained at 50°C for 1 min, then raised to 250°C at a rate of 7°C/min, and maintained in 250°C for 10 min. Helium was used as carrier gas at a column head pressure of 170 kPa. The same parameters were used for all analyses.

2.3. Extraction of T. peregrinus Adults. Extractions were made with mated and virgin males and females of different ages (3–9, 10–21, 22–34 days old), according to availability of insects. Quantified extracts were compared for virgin males and females (3–9 days), virgin and mated males (10–21 and 21–34 days old), and mated males and females (10–20 and 21–34 days old). There were at least two repetitions per treatment, with a minimum of 15 insects extracted in total. In both experiments, insects were separated by sex in glass Erlenmeyer flasks. The flasks with insects were put in a freezer for one hour so that they died with the rectal organ exposed while "glued" to the glass. Thus, the adults were extracted as complete adults with their rectal organ exposed. The extraction was made between 11:00 AM and 16:00 PM using 150 μL of double distilled HPLC-grade hexane for 10 minutes, then 150 μL of a tridecane solution was added as an IS. The samples were concentrated before injection into a GC-2010, a GC-MS-QP 2010 Plus, and a GC-Fourier transform infrared spectroscopy (GC-FTIR) (GC-2010 coupled to a DiscovIR-GC—Shimadzu). In the infrared analysis, the GC was operated in the splitless mode, and equipped with a DB-5 (0.25 μm, 0.25 m × 30 m) (J&W Scientific, Folsom, CA, EUA) capillary column with helium carrier gas. The column oven was maintained at 50°C for 1 min and then increased to 250°C at 7°C/min to 250°C. A liquid-nitrogen-cooled photoconductive mercury-cadmium-telluride (MCT) detector was used with FT-IR resolution of 8 cm^{-1}. As for nymphal extracts, the final concentration of the IS was calculated

for each extract, and extracted compounds were quantified based on the area of the IS.

2.4. Identification of Chemical Compounds and Synthesis of Esters. Compound identifications were based on coinjections with synthetic standards, Kovats indices (KI), mass spectra (MS), and GC-FTIR analysis. Benzaldehyde, octanol, octanoic acid, decanal, hexanoic acid were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). (*E*)-2-octenol was purchased from Acros Organics (Geel, Turnhout, Belgium).

Twenty-one esters were synthesized by esterification of propionic acid, isobutyric acid, and butyric acid with the following alcohols: pentanol, 3-methylbutan-1-ol, 3-methylbut-2-en-1-ol, 3-methylbut-3-en-1-ol, (*Z*)-pent-2-en-1-ol, (*E*)-pent-2-en-1-ol, pent-4-en-1-ol (all from Aldrich Chemical Company, Milwaukee, WI, USA). 3-methyl-2-buten-1-ol (34.03 mmol, 3 g) and butyric acid (68.06 mmol, 6 g) were refluxed in a round-bottom flask with p-toluenesulfonic acid (TsOH) (1 mol%, 0.35 mmol, 0.06 g) and hydroquinone (5% (w/w) in relation to alcohol, 1.75 mmol, 0.191 g). The reaction medium was heated to 60°C with magnetic stirring for 3 h under argon. The product was purified by addition of aqueous solution NaOH 10% (m/V) until the pH was neutral. Afterwards, the product was extracted with ethyl ether; the combined organic solutions were washed with saturated $NaHCO_3$ solution and brine, dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation. The crude product was purified by distillation at reduced pressure and collected from 150–160°C. The same experimental conditions were used in the syntheses of the other saturated esters; however, hydroquinone was not employed with saturated alcohols.

The following esters were coinjected with the natural extracts on three different GC columns (DB-5, DB-Wax and HP-1) for identifications: pentyl propionate (yielding 94%), 3-methylbutyl propionate (yielding 74%), pent-4-en-1-yl propionate (yielding 91%), (*Z*)-pent-2-en-1-yl propionate (yielding 83%), (*E*)-pent-2-en-1-yl propionate (yielding 83%), 3-methylbut-2-en-1-yl propionate (yielding 92%), 3-methylbut-3-en-1-yl propionate (yielding 90%), pentyl 2-methylpropanoate (yielding 90%), 3-methylbutyl 2-methylpropanoate (yielding 87%), pent-4-en-1-yl 2-methylpropanoate (yielding 92%), (2Z)-pent-2-en-1-yl 2-methylpropanoate (yielding 61%), (2E)-pent-2-en-1-yl 2-methylpropanoate (yielding 71%), 3-methylbut-2-en-1-yl 2-methylpropanoate (yielding 92%), 3-methylbut-3-en-1-yl 2-methylpropanoate (yielding 75%), pentyl butyrate (yielding 90%), 3-methylbutyl butyrate (yielding 92%), pent-4-en-1-yl butyrate (yielding 92%), (*Z*)-pent-2-en-1-yl butyrate (yielding 68%), (*E*)-pent-2-en-1-yl butyrate (yielding 74%), 3-methylbut-2-en-1-yl butyrate (yielding 92%), and 3-methylbut-3-en-1-yl butyrate (yielding 80%).

2.5. Statistical Analysis. Statistical analyses were performed using R version 2.13 [10]. To analyze the six main compounds found in the exuviae, the Kruskal-Wallis rank sum test was used followed by a nonparametric multiple comparisons test using the package "pgirmess" in case of significance. Data for the comparison of extracts of virgin and mated adults were tested for normality by the Liliefors and Shapiro-Wilk test. After the normality of the data was confirmed ($P > 0.05$), we performed a GLM (generalized linear model) procedure following Gaussian distribution, considering that mating status was an independent variable. For all analyses, P values >0.05 were considered not significant.

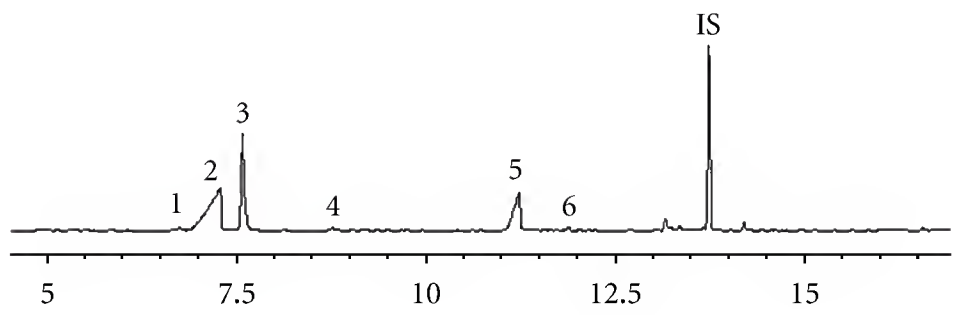

FIGURE 2: Typical gas chromatogram of a *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae) exuvial extract. Numbers (1–6) correspond, respectively, to benzaldehyde, hexanoic acid, octanal, (*E*)-2-octenal, octanoic acid, and decanal. Extracts were analyzed on a Shimadzu GC MS-QP 2010; the internal standard (IS) was tridecane.

3. Results and Discussion

3.1. T. peregrinus Exuvial Extraction. Six compounds were present in the exuviae of *T. peregrinus* nymphs, including benzaldehyde, octanol, (*E*)-2-octenol, octanoic acid, decanal, and hexanoic acid (Table 1) (Figure 2). Fourth and fifth instars produced more hexanoic ($H_4 = 15.9$, P value =0.003) and octanoic acids ($H_4 = 15.9$, P value =0.003) than did first instars. All other compounds did not differ significantly by instar; benzaldehyde ($H_4 = 7.9$, P value =0.09), octanol ($H_4 = 6.1$, P value =0.19), (*E*)-2-octenol ($H_4 = 3.2$, P value =0.52), and decanal ($H_4 = 3.9$, P value =0.41) (Table 1).

Some of the compounds present in the exuviae of *T. peregrinus* have been found in other heteropteran species, either as repellents or attractants. For example, benzaldehyde from copulating pairs of *Triatoma infestans* (Klug, 1834) (Reduviidae) was highly attractive to conspecific females at low doses (0.05–0.1 μg) [11]. In the bed bug, *Cimex lectularius* (Linnaeus, 1758) (Cimicidae), decanal, (*E*)-2-octenal, and benzaldehyde are reportedly essential components of the airborne aggregation pheromone [12]. The hexanoic acid is produced in metathoracic scent gland secretions of many bugs (e.g., Scutelleridae: *Eurygaster maura* (Linnaeus, 1758)), along with (*E*)-2-hexanal, (*E*)-2-hexenyl acetate, *n*-tridecane, octadecanoic acid, and *n*-dodecane [13]. The alarm pheromone of *Leptoglossus zonatus* (Dallas, 1852) (Coreidae) adults includes hexyl acetate, hexanol, hexanal, and hexanoic acid [14]. Also, in Japan, a mixture of (*E*)-2-octenyl acetate and 1-octanol attracted the rice bug, *Leptocorisa chinensis* Dallas, 1852 (Alydidae) [15]. While the compounds identified here for *T. peregrinus* nymphs are commonly known exocrine compounds of Heteroptera, the combination of these compounds in these thaumastocorid nymphs is unique compared to the secretions of other heteropteran nymphs [6]. Other heteropterans produce some of these compounds (e.g., *Cimex lectularius*) but not

Table 1: Identification and quantification (ng) of compounds present in *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae) exuviae. Different letters for each compound indicate significant differences between instars (KI: Kovats Index; SE: standard error). Statistical comparisons: Kruskal-Wallis rank sum test followed by a nonparametric multiple comparisons test ($P > 0.05$).

Chemical compounds	DB-5 column	1st instar	2nd instar	3rd instar	4th instar	5th instar
	KI	Mean SE	Mean SE	Mean SE	Mean SE	Mean SE
(1) Benzaldehyde	962	0.1 ± 0.0^a	0.1 ± 0.0^a	0.2 ± 0.0^a	0.1 ± 0.0^a	0.1 ± 0.0^a
(2) Hexanoic acid	988	0.4 ± 0.0^a	1.7 ± 0.5^a	8.5 ± 3.9^a	21.2 ± 7.8^b	25.9 ± 6.7^b
(3) Octanal	996	2.1 ± 0.6^a	5.9 ± 1.2^a	5.7 ± 1.7^a	6.6 ± 2.6^a	3.6 ± 1.1^a
(4) (*E*)-2-octenal	1062	0.3 ± 0.1^a	0.4 ± 0.1^a	0.3 ± 0.0^a	0.3 ± 0.0^a	0.2 ± 0.1^a
(5) Octanoic acid	1209	0.2 ± 0.0^a	0.9 ± 0.3^a	2.6 ± 1.1^a	7.4 ± 2.9^b	7.3 ± 0.8^b
(6) Decanal	1239	0.3 ± 0.1^a	0.4 ± 0.1^a	0.3 ± 0.2^a	0.2 ± 0.1^a	0.1 ± 0.0^a
Total (ng)		3.4	9.5	17.7	35.7	37.2

all of them combined. The significance of this uniqueness is unknown.

3.2. Extracts of T. peregrinus Adults. The chromatographic profiles of extracts from *T. peregrinus* males and females revealed the presence of two esters, one minor (A) and one major (B). Their retention times (Rts) and Kovat's Indices (KIs) on the RTX-5 column were as follows: **A**: Rt = 8.867 min., KI = 1068 and **B**: Rt = 9.558 min, KI = 1103. The MS of **B** showed a base peak at *m*/*z* 71, fragments at *m*/*z* 68, *m*/*z* 85, *m*/*z* 128, and a molecular ion of 156 Da (Figure 3). When this spectrum was compared to the NIST library, it was evident that **B** might be a propionic, isobutyric, or butyric ester, with a molecular formula of $C_9H_{16}O_2$. The most important signals in the GC-FTIR spectrum of **B** (Figure 3) were a C-H vibration band (2962; 2935; 2871 cm^{-1}), an ester carbonyl band (1730 cm^{-1}), and multiple bands of C(CO)O characteristic of esters (1445, 1381, and 1189 cm^{-1}). These bands associated with a band of C–H stretching vibration of substituted double bond in 3023 cm^{-1}, and the presence of a band in 1674 cm^{-1}, characteristic of trialkyl-substituted alkenes, showed that compound **B** was an unsaturated ester with an internal double bond. In contrast, the molecular ion in MS of compound **A** was not obvious; however, the base peak at *m*/*z* 68 and a fragment at *m*/*z* 71 suggested **A** was an ester similar to **B**. Although the GC-FTIR spectra of **A** showed the same characteristic bands for esters that were detected for **B**, the presence of a band at 3080 cm^{-1} due to asymmetric stretch of a terminal double bond, demonstrating that **A** was an unsaturated ester with a terminal double bond. To positively identify the natural products **A** and **B**, the twenty-one above-mentioned esters were synthesized. Thus, the major compound **B** was identified as 3-methylbut-2-en-1-yl butyrate by coinjection of this standard with the natural extract on the three GC columns (RTX-5, RTX-WAX, and HP-1). Identification was based on coelution and MS. Additionally, the minor compound **A** was identified as 3-methylbut-3-en-1-yl butyrate by coinjection of this standard with the natural extract on the different GC columns.

Females and males produced the same esters, but their quantities varied by sex and age, particularly for the major compound, 3-methylbut-2-en-1-yl butyrate (Figure 4). Although the concentration of the esters in males increased with age (Table 2), reaching a maximum of approximately 1 μg per insect in 22-day-old mated males, this age difference could not be detected statistically. Only the amount of the major compound (**B**) of mated males was statistically different from that for mated females ($F_{1,3} = 10.3$, P value =0.048) (GLM). Ester concentrations of virgin males and females were not statistically different (GLM) for either the minor (**A**) ($F_{1,4} = 0.6$, P value =0.47) or major (**B**) ($F_{1,4} = 3.2$, P value =0.14) compounds. Likewise, ester concentrations of mated and virgin males (**A:** $F_{1,3} = 2.4$, P value =0.21; **B:** $F_{1,3} = 5.7$, P value =0.09), and of mated males and females (minor $F_{1,3} = 4.5$, P value =0.12) were not statistically different (GLM) (Table 2). The adults of 10–21 days old did not have enough repetitions to be compared. Thus, they were not considered for the concentration analysis.

Butyrates and isobutyrates are pheromone components for other Heteroptera, such as broad-headed bugs (Alydidae) [16], plant bugs (Miridae) [17, 18], and predacious stink bugs (Pentatomidae: Asopinae) [19]. Mirid bugs, particularly species of the genus *Phytocoris*, produce unsaturated butyrate and acetate semiochemicals. *Phytocoris* females attract males with sex pheromones based on butyrate and acetate blends [20–22], while males apparently release high concentrations of certain butyrates as antisex pheromones [17]. In *Alydus eurinus* (Say) (Alydidae), the sex pheromone of females is a blend of 2-methylbutyl butyrate and (*E*)-2-methyl-2-butenyl butyrate [16].

The biological function(s) of 3-methylbut-2-en-1-yl and 3-methylbut-3-en-1-yl butyrates in *T. peregrinus* remain to be elucidated. An aggregation function was attributed to the major compound through olfactometer experiments, in which males attracted only males (Gonzalez et al. 2012 this issue); however, we did field tests using delta traps with different concentrations of the major compound, and they all failed to attract insects in the field and in a greenhouse with a *T. peregrinus* population. Allomones and pheromones known for other heteropterans, such as those described above, undoubtedly originate from the dorsal abdominal glands of nymphs or the metathoracic scent glands that are characteristic of most true bug adults. In *T. peregrinus*, however, the metathoracic scent glands are vestigial. The butyrates from *T. peregrinus* appear to be associated with extrusion of the rectal organ (Figure 1) that has

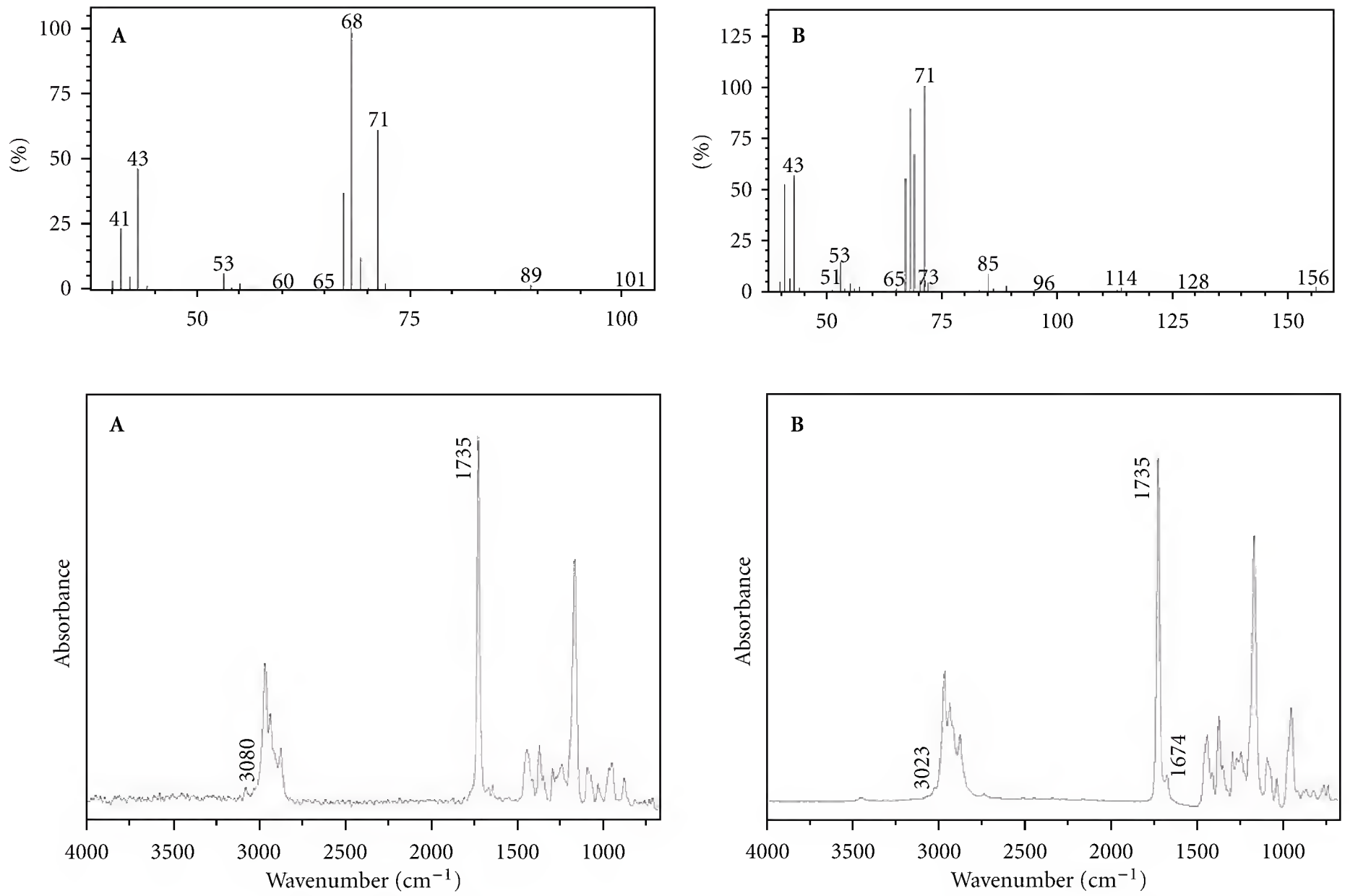

FIGURE 3: Mass and infrared spectra of the minor compound **A** and major compound **B** found in adults of *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae). Extracts were analyzed on a Shimadzu GC MS-QP 2010 and GC-Fourier transform infrared spectroscopy (GC-FTIR) GC-2010 coupled to a DiscovIR-GC—Shimadzu.

TABLE 2: Quantification (μg) of esters identified in whole body extracts of *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae) virgin and mated males and females of different ages; 3–9 days (1) and 22–33 days (3). For each ester identified, ns: not statistically different, and $*$: statistically different; SE: standard error. Statistical comparisons: GLM (generalized linear model) with Gaussian distribution ($P > 0.05$).

		3-Methylbut-3-en-1-yl butyrate (A)		3-Methylbut-2-en-1-yl butyrate (B)
		Mean SE		Mean SE
Virgin males (1)	ns	0.1 ± 0.0	$*$	11.2 ± 3.8
Virgin females (1)		0.2 ± 0.1		0.5 ± 0.0
Virgin males (3)	ns	8.3 ± 0.2	$*$	191.8 ± 19.1
Mated males (3)		20.7 ± 5.4		743.5 ± 61.7
Mated males (3)	$*$	20.7 ± 5.4	$*$	743.5 ± 61.7
Mated females (3)		0.1 ± 0.1		0.5 ± 0.3

heretofore only been described within the Heteroptera for plant bugs (Miridae) [8]. Unequivocal verification that the rectal organ tissue is the source of these esters awaits further experimentation. Mated *T. peregrinus* males produce greater quantities of both esters, especially ester **B**, compared with virgin males and younger mated males. Moreover, these esters are produced by females, suggesting that these compounds are not involved in aggregating the sexes for mating. Speculating the differences of concentration, these esters could be indicators of sex and age recognition by conspecifics.

4. Conclusion

Benzaldehyde, octanol, (*E*)-2-octenol, octanoic acid, decanal, and hexanoic acid were present in the exuviae of *T. peregrinus* nymphs. Volatiles from adult males and females included 3-methylbut-3-en-1-yl butyrate and 3-methylbut-2-en-1-yl butyrate. Compounds identical or similar to those found in *T. peregrinus* exuviae and esters identified in the adults were found in other heteropterans with various functions. The possible pheromonal roles of these volatile blends are being studied.

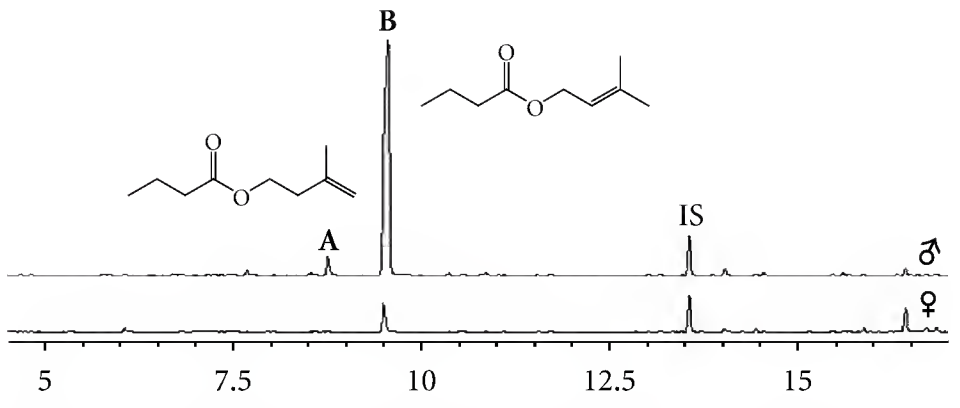

FIGURE 4: Representative gas chromatograms of body extracts of 21-day-old *Thaumastocoris peregrinus* (Heteroptera, Thaumastocoridae) males and females. Minor and major compounds, 3-methylbut-3-en-1-yl butyrate (**A**) and 3-methylbut-2-en-1-yl butyrate (**B**), respectively. The internal standard (IS) was tridecane; extracts shown here were analyzed on GCMS-QP 2010 Plus.

Acknowledgments

The authors thank Renata Morelli for the statistical analysis, Rede Paranaense de Coleções Biológicas in the Universidade Federal do Paraná for the fotos, the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Pessoal de Aperfeiçoamento de Nível Superior (CAPES), and the Semioquímicos na Agricultura (INCT) for the financial support.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 916718, 7 pages
doi:10.1155/2012/916718

Review Article

Descriptions of New Species of Leucotrichiinae (Trichoptera: Hydroptilidae) from Brazil

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Received 13 February 2012; Accepted 27 March 2012

Academic Editor: Ai-Ping Liang

Two new species of Hydroptilidae (Trichoptera) from Brazil are described: *Betrichia alibrachia* sp. n. and *Leucotrichia bicornuta* sp.n. Both genera are members of the subfamily Leucotrichiinae. Illustrations of male genitalia are provided with each description. These additions bring the total world fauna of *Betrichia* to 9 species and *Leucotrichia* to 29 species.

1. Introduction

The subfamily Leucotrichiinae (type genus *Leucotrichia* Mosely, 1934) was established by Flint (1970) and currently contains 16 genera: *Abtrichia, Acostatrichia, Alisotrichia, Anchitrichia, Ascotrichia, Betrichia, Celaenotrichia, Cerasmatrichia, Ceratotrichia, Costatrichia, Eutonella, Leucotrichia, Mejicanotrichia, Peltopsyche, Scelobotrichia*, and *Zumatrichia* [1, 2]. The distribution of the subfamily is limited to the New World and found predominantly in Central America [3, 4].

Mosely (1939) established the genus *Betrichia* for a single species, *Betrichia zilbra*, from Brazil (Santa Catarina) [5]. No exact reasons were stated, but presumably the genus was erected on account of venational, antennal, and general male genitalic features [3]. Additional species and distributions have been provided by Angrisano, Flint, and Olah and Johanson [6–10]. Up to and including species described by Olah and Johanson in 2011, *Betrichia* contained a total of 8 extant species (Table 1) [2, 10]. As a genus, its distribution is limited to eastern South America (Table 1) [4]. Immature stages are unknown [3]. Adults are attracted to lights and can generally be found near lowland rivers [4].

Mosely (1934) established the genus *Leucotrichia* for a single species, *Leucotrichia melleopicta*, from Mexico (Tabasco) [11]. Additional species descriptions and distributions have been provided by Angrisano and Burgos, Banks, Botosaneanu (in Botosaneanu and Alkins-Koo), Bueno-Soria, Bueno-Soria et al., Flint, Olah and Johanson, Ross, Rueda Martín, Sattler and Sykora, and Wells and Wichard [1, 10, 12–23]. Up to and including species described by Olah and Johanson and Rueda Martín in 2011, *Leucotrichia* contained a total of 28 extant species (Table 1) [2, 10, 20]. The distribution of the genus includes most of the continental USA, the Greater and southern Lesser Antilles, Central America, and northern South America (Table 2) [4]. A single fossil species, *Leucotrichia adela*, is known from Dominican amber [22]. Adults occasionally come to light but are usually taken by net during the day from marginal foliage [4].

In this paper, I describe two new species, *Betrichia alibrachia* and *Leucotrichia bicornuta*, from Brazil. This brings the total world fauna of *Betrichia* to 9 and of *Leucotrichia* to 29.

2. Taxonomy

2.1. Leucotrichiinae Flint, 1970. Past attempts at dividing Hydroptilidae into suprageneric units based primarily on larval morphology generally have produced groups of genera that were not or could not be placed into easily distinguishable groups [1]. *Leucotrichia* and its closely related genera have often proven to be a somewhat aberrant unit in these attempts. Flint stated that, although there was no single characteristic that could be used to consistently separate

Table 1: Species and distributions of *Betrichia* and *Leucotrichia*.

Species	Distribution
Betrichia	
argentinica Flint 1972	Argentina, Uruguay
bispinosa Flint 1974	Surinam
hamulifera Flint 1983	Argentina, Paraguay, Uruguay
longistyla Flint 1983	Brazil
occidentalis Flint 1974	Surinam
rovatka Olah and Johanson 2011	French Guiana
uruguayensis Angrisano 1995	Uruguay
zilbra Mosely 1939	Brazil, Uruguay
Leucotrichia	
alisensis Rueda-Martín 2011	Argentina
ayura Flint 1991	Colombia
botosaneanui Flint 1996	Tobago, Trinidad
brasiliana Sattler and Sykora 1977	Brazil
brochophora Flint 1991	Colombia
chiriquiensis Flint 1970	Panama
dinamica Bueno-Soria 2010	Mexico
extraordinaria Bueno-Soria et al. 2001	Mexico
fairchildi Flint 1970	Colombia, Grenada, Panama, Tobago, Trinidad, Venezuela
forrota Olah and Johanson 2011	Peru, Ecuador
gomezi Flint 1970	Dominican Republic
imitator Flint 1970	Costa Rica, Guatemala, Mexico
inflaticornis Botosaneanu 1993	Trinidad
inops Flint 1991	Colombia
interrupta Flint 1991	Colombia
laposka Olah and Johanson 2011	Peru
lerma Angrisano and Burgos 2002	Argentina
limpia Ross 1944	Mexico, U.S.A.
melleopicta Mosely 1934	Mexico, Venezuela
mutica Flint 1991	Colombia
padera Flint 1991	Colombia
pictipes Banks 1911	U.S.A., Mexico
sarita Ross 1944	Costa Rica, El Salvador, Guatemala, Mexico, U.S.A.
termitiformis Botosaneanu 1993	Trinidad
tritoven Flint 1996	Tobago, Trinidad, Venezuela
tubifex Flint 1964	Dominican Republic, Haiti, Jamaica, Puerto Rico
viridis Flint 1967	El Salvador, Guatemala, Mexico, Panama
yungarum Angrisano and Burgos 2002	Argentina

adults from other subfamilies, a combination of characters were diagnostic for Leucotrichiinae when present [1]. These characters included the reduction of ocelli to 2, the presence of modified setae, the presence of a basal costal pouch on the forewing, and something characteristic in the form of the male genitalia that was hard to define exactly [1], but likely includes the "window" and basal loop medially on the phallic apparatus. The character that was absolutely distinctive and primarily responsible for Flint's establishment of Leucotrichiinae was the larval shelter; the shelter is no more than a dorsal covering nor is it moveable, characteristics not possessed by any other genus [1].

In her revision of the genera of Hydroptilidae, Marshall recognized only two subfamilies: Ptilocolepinae and Hydroptilinae [3]. Leucotrichiini was included within Hydroptilinae, due to the many features characteristic of Hydroptilinae in general [3]. Marshall also stated that although Leucotrichiini does form a distinct group within Hydroptilinae, it was

Table 2: Species groups of *Leucotrichia*.

L. melleopicta species group	*L. pictipes* species group	*Incertae sedis*
L. ayura	*L. fairchildi*	*L. alisensis*
L. brochophora	*L. imitator*	*L. botosaneanui*
L. chiriquiensis	*L. pictipes*	*L. brasiliana*
L. dinamica	*L. sarita*	*L. forrota*
L. extraordinaria		*L. laposka*
L. gomezi		*L. lerma*
L. inflaticornis		*L. mutica*
L. inops		*L. termitiformis*
L. interrupta		*L. tritoven*
L. limpia		*L. yungarum*
L. melleopicta		
L. padera		
L. tubifex		
L. viridis		

impossible to exactly define the genera, since species from two or more genera often key out with one another in generic-level keys [3].

Malicky elevated Ptilocolepinae to family rank Ptilocolepidae, leaving only one subfamily of six tribes in Hydroptilidae [24]. This effectively raised each tribe back to the subfamily status originally designated by Flint.

2.2. Betrichia Mosely, 1939. In the original description, Mosely mentioned the presence of ocelli (the type-specimen bears 2), short antennae with a long basal joint, elongate wings with acute produced apices, and a 1, 3, 4 tibial spur formula as being part of the diagnosis of the genus [5]. Maxillary palpi were missing from the holotype, the single specimen that was examined, and so was unavailable for description. Genitalic features and a forewing measurement of 3 mm were included in the species description of *B. zilbra* [5]. Among the genitalic features mentioned were the deeply excised apical margin of tergite VIII, the shallowly excised apical margin of tergite IX with apical angles produced into a pair of irregular rounded lobes, a phallus with a pair of stout sheaths, and a slender process on sternite VII [5]. Wing venation and male genitalia were illustrated in the description of the type-species [5].

In her review of the genera of Hydroptilidae, Marshall concluded that the combination of features given in the original generic description was not unique to *Betrichia* and that there are no precise diagnostic features that distinguish it from other genera [3, 4]. Of the additional species added to the genus, most differ noticeably in some way from the type-species. For example, *Betrichia argentinica* shares similar genitalic features with the type-species, but also bears 3 ocelli and simple antennae. The male genitalic features of *Betrichia surinamensis* differ greatly from that of the type-species and *Betrichia bispinosa* bears a basal costal pouch not found throughout the genus. Flint has stated that the establishment of additional genera to sort out such variation should wait until the South American Trichoptera fauna is better understood [8]. According to Marshall, all small leucotrichiine genera, such as *Betrichia*, should be placed in a group together, as characters originally diagnosed as generic features have proven to be specific as additional species have been described [3]. Marshall's summary of male genitalic features reflects the variation now found within the genus, including features such as a variable subgenital plate, variable tergite X, and sternite VIII and segment IX each with or without posterolateral processes [3].

2.3. Leucotrichia Mosely, 1934. In the original description, Mosely mentioned the presence of ocelli, antennal joints grouped in series of white and brown, a well-developed frenulum on the hindwing, arising from the subcosta, very densely setose legs, and a 1, 3, 4 tibial spur formula as being part of the generic diagnosis [11]. Maxillary palpi were mentioned as difficult to observe. Male genitalic features and a forewing measurement of 2 mm were included in the species description of *L. melleopicta* [11]. Among the genitalic features mentioned were the flattened terminal dorsal segment covering the genitalia, the deeply excised terminal ventral segment bearing setose margins, a spade-shaped "penis sheath", and a slender process on the penultimate abdominal segment [11]. Wing venation and male genitalia were illustrated in the description of the type-species [11].

As additional new species were described and placed in *Leucotrichia*, Flint noted that, despite variation in antennae, number of ocelli, and dorsal structure of the head, there were certain other features that could be used to unite 2 species groups within the genus [1]. Characters that Flint found distinguished the *L. melleopicta* species group include males bearing 3 ocelli, a mesoventral process on sternum VII, an unmodified head (except for *L. chiriquiensis*), and 1 or 2 large areas of greenish or whitish setae on the forewings [1]. Characters that Flint found distinguished the *L. pictipes* species group include males bearing 2 ocelli, either a brush of setae or a point on sternum VII, a modified head (except for *L. imitator*), and spots or linear greenish or whitish marks on the forewings [1].

Flint also provided a generic-level diagnosis, including additional characters not in the original diagnosis. Mentioned, among other features, were the pentagonal metascutellum, the heavily sclerotized trianguloid plate of tergite X, the subgenital plate extending ventrally from the ventral angles of tergite X to the base of the inferior appendages, the subapical spine of the inferior appendages, and the midlength complex of the phallus [1]. Flint did not mention the wings in his generic diagnosis, while Marshall stated in her review of hydroptilid genera that the wings are unmodified, but made no mention of a well-developed frenulum on the hindwing [1, 3].

Larvae have been associated with some species in the genus, but many are still unassociated [4]. The larvae are generally typical of the Leucotrichiinae but can be distinguished by their rugose or papillate head, single tarsal claw, and femora bearing spiniform dorsal setae [3]. The larvae, which are strongly depressed and may reach up

to 5 mm in length, are free-living and found in running water on the upper surface of rocks, grazing on periphyton, until the final instar [25, 26]. During this final retreat-dwelling instar, they also display extreme lateral distention of abdominal segments V–VII [26]. The silken retreats are generally flattened ovals up to 5.5 mm in length, slightly domed, with a circular opening at each end, attached tightly to rocks in fast flowing waters [3, 4, 26].

3. Materials and Methods

Morphological terminology used for male genitalia of specimens follows that of Marshall [2]. For simplicity, paired structures are discussed in the singular. Procedures for specimen preparation followed those explained in detail by Blahnik et al. [27]. For specimen examination and illustration, cleared genitalia were placed in a watch glass with glycerin and small glass beads. The glass beads held the genitalia in place and allowed structures to be viewed in precise lateral, dorsal, and ventral positions. Genitalia were examined with an Olympus BX41 compound microscope at 250–500x magnification. Structures were traced in pencil with the use of an Olympus model U-DA drawing attachment mounted on the microscope. Pencil sketches were then scanned (Fujitsu ScanSnap S1500M scanner), edited in Adobe Photoshop (v. 9.0.2, Adobe Systems Inc.), and used as a template in Adobe Illustrator (v. 13.0.2, Adobe Systems Inc.) to be digitally inked. Electronic "drawing" was completed with the aid of a graphics tablet (Bamboo Fun, Wacom Company, Limited). Illustrations shown in Figures 1 and 2 were produced from holotype specimens. Species descriptions were constructed using the program DELTA [28] and specimen management followed the procedures outlined by Holzenthal and Andersen [29]. Individual specimens examined during the study were associated with a barcode label (4 mil polyester, 8 × 14 mm, code 49) bearing a unique alphanumeric sequence beginning with the prefix UMSP. Vials of alcohol containing multiple specimens were given a single barcode label to represent all those in the vial. The prefix is not meant to imply ownership by the University of Minnesota Insect Collection (UMSP), but only to indicate that the specimen was databased at that collection. Types of species described and other material examined are deposited in the Coleção Entomológica Professor José Alfredo Pinheiro Dutra, Departamento de Zoologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro (DZRJ), and the University of Minnesota Insect Collection, St Paul, Minnesota (UMSP).

4. New Species Descriptions

4.1. *Betrichia alibrachia sp.n. (Figure 1)*

Material. Holotype male: Brazil: Rio de Janeiro: Resende, Ribeirás do Palmital, 22°25′26.2″S, 44°44′21.6″W, 969 m, 8.iii.2008, white light, collected by Nessimian, Dumas, de Souza, and Braga (in alcohol) (UMSP0000140290) (DZRJ). *Paratypes:* same data as holotype (9 males) (in alcohol) (DZRJ); Resende, Riacho no km 4 BR 354, 22°23′20″S, 44°45′16″W, 1411 m, 9.iii.2008, white light, collected by Nessimian, Dumas, de Souza, and Braga (1 male) (in alcohol) (DZRJ); Itatiaia, Parque Nacional do Itatiaia, ponte no Lago Azul, 22°27′02.8″S, 44°36′50.2″W, 29.viii.2009, UV light, collected by Cardosio-Costa and de Souza (1 male) (in alcohol) (DZRJ); Resende, Ribeirás do Palmital, 22°25′26.2″S, 44°44′21.6″W, 969 m, 8.iii.2008, UV light, collected by Nessimian, Dumas, de Souza, and Braga (2 males) (in alcohol) (UMSP); Nova Friburgo, Macaé de Cima, Rio Macaé, 22°24′46.0″S, 42°31′16.2″W, 13.ix.2009, collected by Santos (1 male, 1 female) (in alcohol) (DZRJ).

Description Male. Length of forewing 1.8–2.6 mm (n = 9). Head unmodified, with 3 ocelli; antennae unmodified. Wings unmodified. Tibial spur count 1, 3, 4. Color in alcohol brown, denuded. *Genitalia.* Abdominal sternum VII mesoventral process acute. Segment VIII anterolateral margin acute, posterolateral margin with 3 projections, 1st and 3rd slender, elongate with single prominent seta, 2nd less pronounced, with single seta; in ventral view posterior margin acutely concave. Segment IX anterolateral margin with 2 pairs of elongate apodemes extended anteriorly, as in Figures 1(a) and 1(c), posterolateral margin setose, rounded; with elongate mesolateral rasp-like structure within membranous layer, extended anteriorly into segment VI, with triangular sclerotized structure at base of membranous layer, apex curving dorsad; dorsally segment IX with anterior margin concave. Subgenital plate not apparent. Inferior appendage apparently fused basally to segment IX, elongate, quadrate basodorsally, apex subacute, extended posteriorly further than tergum X. Tergum X membranous, rounded dorsally, apex emarginate in lateral view. Phallus tubular basally, with median complex bearing basal loop and pair of circular "windows," apex with rounded ventral membranous projection.

Diagnosis. This species has several features that distinguish it from all other species in the genus. It lacks both an apparent subgenital plate and apical spines or sclerites on the phallus. It also bears an elongate rasp-like process extending anteriorly from segment IX. This process is not present elsewhere in the genus, or in any other genera of which I am aware.

Etymology. Alius, Latin for "another"; *brachium*, Latin for "arm", referring to the very unusual rasp-like structure extending anteriorly from segment IX.

4.2. *Leucotrichia bicornuta sp.n. (Figure 2)*

Material. Holotype male: Brazil, Rio de Janeiro, Panedo, Rio das Pedras, Três Bacias, 22°24′32.2″S, 44°33′06.5″W, 735 m, 6.iii.2008, collected by Nessimian, Dumas, de Souza, and Braga (in alcohol) (UMSP000014084) (DZRJ). *Paratypes*: Brazil, Rio de Janeiro, Itatiaia, Parque Nacional do Itatiaia, Rio Camp Belo, 22°27′17.32″S, 44°36′37.47″W, 705 m, 13.iv.2007, light, collected by Santos, Dumas, Ferreira, Jr., and Nessimian (2 males, 1 female) (in alcohol) (DZRJ).

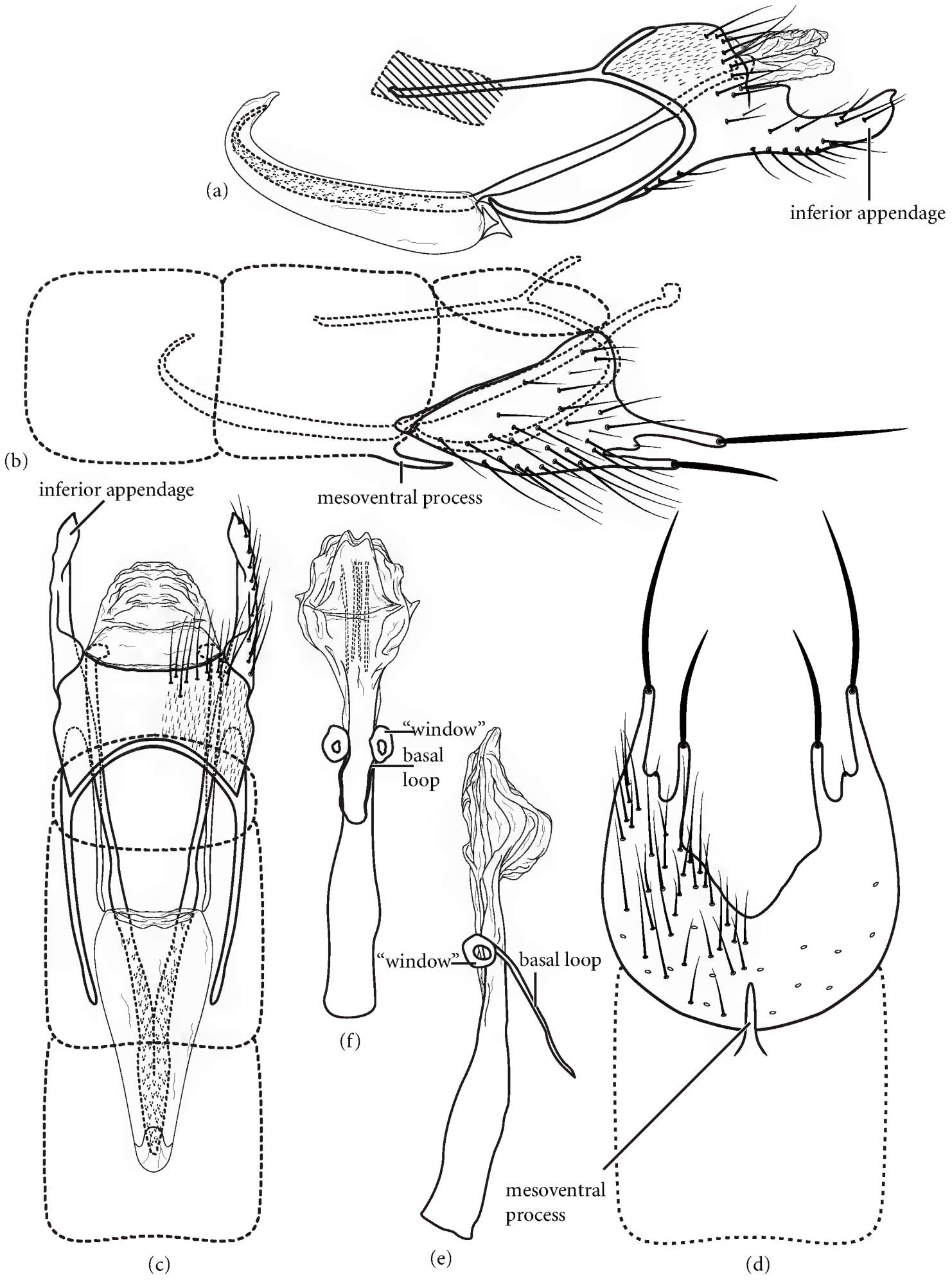

FIGURE 1: *Betrichia alibrachia* sp.n., male genitalia: (a) segments IX-X, left lateral (base of phallus crosshatched); (b) segments VI–VIII and segment IX anterolateral margin, left lateral; (c) segments VI–X, dorsal; (d) segments VII-VIII, ventral; (e) phallus, left lateral; (f) phallus, dorsal.

Description Male. Length of forewing 2.5–3.4 mm ($n = 3$). Head unmodified, with 3 ocelli; antennae unmodified. Tibial spur count 1, 3, 4. Color in alcohol brown, denuded. *Genitalia*. Abdominal sternum VII mesoventral process basally broad, rounded apically. Segment VIII anterolateral margin convex, posterolateral margin with single rounded projection bearing prominent setae; in ventral view posterior margin broadly concave. Segment IX anterolateral margin broadly produced dorsolaterally, posterolateral margin setose, broadly convex; dorsally with anterior margin broadly convex. Subgenital plate extending from ventral angle of tergum X, ventral arm extending to base of inferior appendage. Inferior appendage without setae, narrow basally, not extended posteriorly past tergum X, with small spine dorsally, apex curving dorsad, in ventral view apex with rounded emargination. Tergum X with semielliptic sclerite

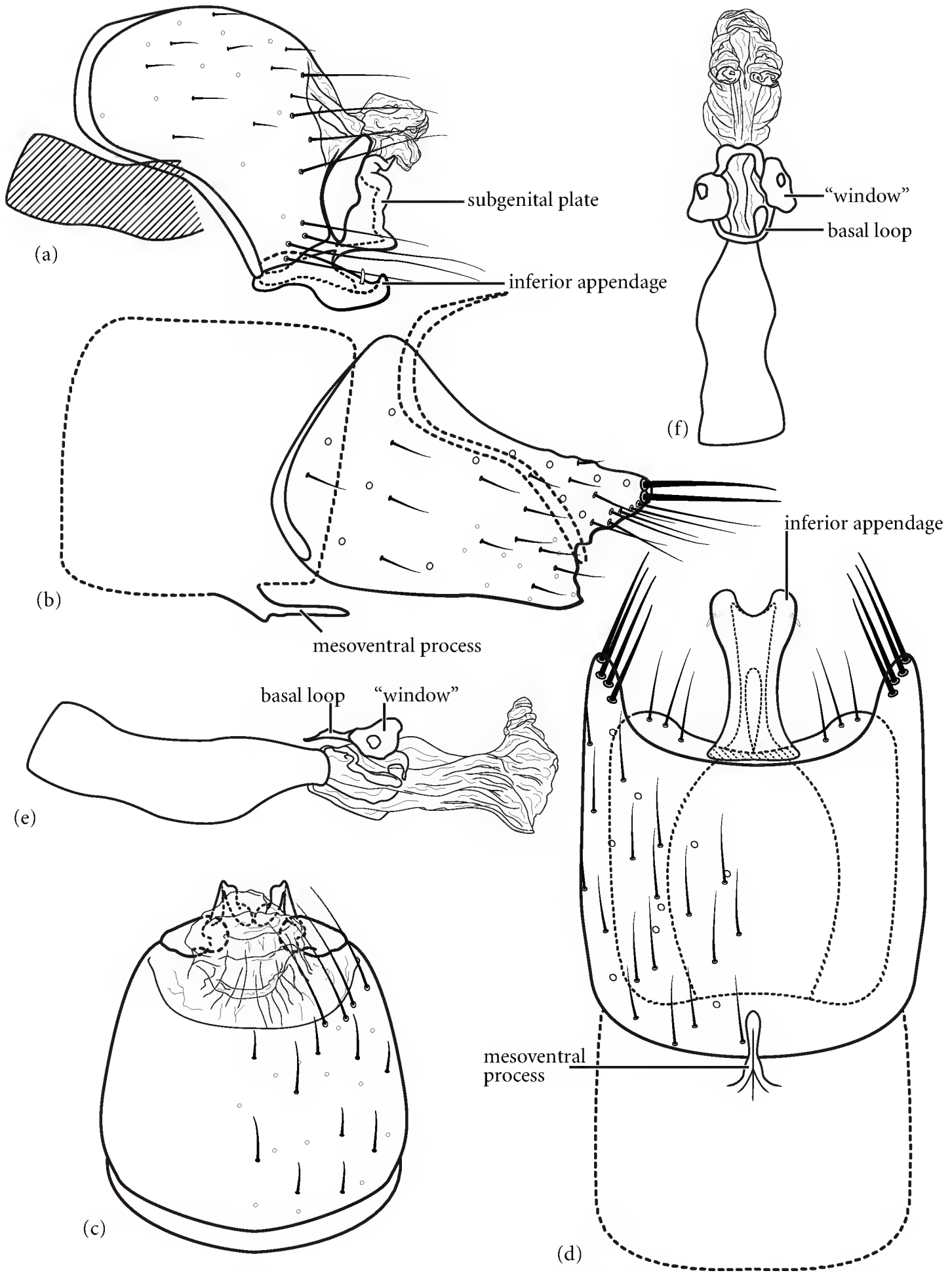

FIGURE 2: *Leucotrichia bicornuta* sp.n., male genitalia: (a) segments IX-X, left lateral (base of phallus crosshatched); (b) segments VII-VIII and segment IX anterolateral margin, left lateral; (c) segments IX-X, dorsal; (d) segments VII–IX, ventral; (e) phallus, left lateral; (f) phallus, dorsal.

with tridentate posterior margin; with membranous suborbicular projection apically. Phallus tubular basally, with median complex bearing basal loop and pair of circular "windows," membranous apex bearing paired dorsal lobes.

Diagnosis. This species is most similar to *Leucotrichia ayura* Flint, 1991, a member of the *Leucotrichia melleopicta* species group [18]. *Leucotrichia bicornuta* is distinguished by the ventral arm of the subgenital plate, which does not project as far posteriad as that of *L. ayura* and then bends dorsad instead of remaining straight. *Leucotrichia bicornuta* is also distinguished by the lack of dorsal sclerites or apical spines on the phallus. Also, in *L. bicornuta*, the inferior appendages are broadly fused mesally; in *L. ayura* they are separate.

Etymology. *Bi*, Latin for "double"; *cornutus*, Latin for "horned", referring to the 2 apicodorsal lobes of the phallus.

Acknowledgments

The author is grateful to Allan Paulo Moreira Santos and Jorge Luiz Nessimian, Universidade Federal do Rio de Janeiro

for permission to describe the new species collected during their research in Brazil. Dr. OS Flint, Jr., Smithsonian Institution, Dr. RW Holzenthal, University of Minnesota, and 2 anonymous reviewers provided invaluable comments on the identity and placement of these new species and reviewed the paper. This material is based on work supported by the National Science Foundation Grant no. 0816865 to KM Kjer and RW Holzenthal. The author was supported also by a CIC Smithsonian Institution Fellowship. This support is gratefully acknowledged.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 267361, 6 pages
doi:10.1155/2012/267361

Research Article

Extreme Effects of Season on the Foraging Activities and Colony Productivity of a Stingless Bee (*Melipona asilvai* Moure, 1971) in Northeast Brazil

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Received 5 March 2012; Revised 13 April 2012; Accepted 15 April 2012

Academic Editor: James Charles Nieh

This study reports the influence of season on foraging activities and internal colonial parameters of *Melipona asilvai* in an Atlantic forest area of northeast Brazil. We used video cameras connected to a PC to monitor all departures and returns of foragers and the types of materials they carried. Foraging activities decreased almost 90% from dry to rainy seasons, but temperature and humidity were not the main factors influencing departures. Observed honey storage and an extreme cutback in activities during the rainy period suggest a seasonal diapause in this species.

1. Introduction

Foraging activities in social insects are influenced by unpredictable environmental variables in terms of timing and location of food [1]. According to Biesmeijer and de Vries [2], there are two main features which govern foraging activities of bees: (1) internal factors, such as individual memory and threshold response to react to the foraging stimuli, and (2) external factors, such as environmental and colony conditions which determine the level of exposure to stimuli associated with the decision [3–8]. Colonies of honeybees and stingless bees can allocate more foragers to collect nectar and pollen in response to the amount of food in storage and availability of resources in the field [7, 9–12].

Stingless bee colonies consist of several hundred to tens of thousands of individuals, and information exchange among the workers is a key feature to colony foraging efficiency and indirectly to colony growth and reproductive success [13]. The influence of weather on foraging activities has been studied in several eusocial bee species [14–25]. These studies report that weather conditions, light intensity, humidity, food availability, competition, colony state, and physiological conditions of individuals are important factors that influence the foraging activities of *Melipona* species.

In this study we report an extreme effect on foraging activity and colony production in response to environmental variables for colonies of *Melipona asilvai*. For this purpose, we used a novel observational approach in order to monitor all daily departures and entrances of foraging bees.

2. Material and Methods

2.1. Study Site. The experiments were performed at the Campus of Universidade Federal de Sergipe (UFS), São Cristóvão (10°55′S, 37°03′W, altitude 2 m). The study area is characterized as a subhumid area of Atlantic Rain Forest or "Zona da Mata." According to Amâncio [26], two distinct seasons are found in this region: a rainy season happening from April to August (pluviosity between 1.100 mm and 1.500 mm) and a dry season taking place from September to March. The air temperature cycle is close to uniform with no significant seasonal thermal variation.

2.2. Species. Three queenright colonies of *Melipona asilvai* were collected for this study. The colonies, originally from

Nossa Senhora da Glória, Sergipe state, were transferred to the UFS Entomology Laboratory. Each colony was housed in a wooden box covered with glass to facilitate observation. A plastic tube connected the colonies to the outside environment, thus permitting the bees to forage freely. The temperature in the hives was controlled at 28°C by means of a thermostat.

2.3. Data Collection. This study was carried out on March 10–28th 2009 (rainy season) and on June 10–28th 2009 (dry season). We used security microcameras (model CCD Sony 480L Day 0.1 Lux Color) which were placed on small glass-covered boxes (5.0 × 3.0 × 3.0 cm) connected to each entrance tube. Video recordings were programmed to start at 05:00 h, before the first foraging departure, and the recording concluded at 19:00 h, after the termination of outside activities. The cameras were linked to a computer using an AVerMedia EZmaker frame grabber (Avermedia, Milpitas, CA) and VirtualDub software, http://www.virtualdub.org/. This setup allowed the observer to identify the corbiculae load, such as mud (irregular-shaped brown material), resin (brighter rounded material), pollen (whitish to yellowish opaque load), and liquid load (water and nectar). Incoming foragers with liquid presented expanded abdomens compared to other unloaded foragers. Nectar and water loads were not individually determined.

To investigate how seasonality affects food storage and colony conditions, we daily counted honey and pollen pots, brood cells in construction, and the relative number of individuals in the colony (workers on the brood combs). All parameters were registered around 18:00 h after video recording. Data on temperature and relative humidity were measured with a digital thermohygrometer kept outside the laboratory.

2.4. Data Analyses. The data were analysed with a general linear model (GLM) where colonies, season, and time of day were entered into the analysis as the independent variables and number of bees entering or exiting as the dependent variables [27]. The Kruskal-Wallis test and the Mann-Whitney U test were used to verify whether the type of load collected by foragers occurred at distinct periods of the day and to compare colony productivity between seasons, respectively. A Kendau Tau correlation test was also used to estimate the relationship between abiotic factors and the frequency of flights. All analyses were made with Statistica 7.0 (Statsoft inc.).

3. Results

3.1. Foraging Activities and Seasonality. General linear mixed models showed that foraging activities were significantly affected by almost all parameters tested (Table 1). Variance between colonies was not significant, meaning that the number of foraging departures and returns between the three colonies were not different. Footage analyses of 73,375 flight returns showed conspicuous differences in activities between rainy and dry seasons. Season, time, and time × season showed significant effects on the frequency of foraging activities.

Table 1: Results of GLM of foraging activities related to dry and rainy seasons, time of day, and studied colonies.

	D.F.	Deviance	F	P
Model	1	932658.2	320.35	0.003
Season	1	805939.7	1019.69	<0.0001
Time	12	9770.8	12.36	0.001
Colony	2	1911.7	2.78	0.06
Season∗time	12	8351.4	10.56	<0.0001
Error	5851	790.4		

There was not a strong correlation of air temperature and relative humidity with the frequency of foragers' exiting (Figure 1; dry season: temperature: $\tau = -0.20$, $P = 0.83$ and humidity: $\tau = 0.34$, $P = 0.73$; rainy season: temperature: $\tau = 2.71$, $P < 0.05$ and humidity:$\tau = -0.03$, $P = 0.37$). On the other hand, a comparison of pooled data showed a positive tendency between temperature and number of bees exiting the nest ($\tau = 13.94$, $P < 0.001$).

3.2. Foraging for Resources, Time of Day, and Season. The onset of nest departures during the dry season occurred around 5:30 h. During the rainy season, the first exiting trips started between 6:00 and 9:00 h, with an exceptional initial foraging exit occurring at 13:00 h. In both seasons, foraging trips ended around 18:00 h. During the dry season, the activity peak of departures occurred between 7:00 and 8:00 h (mean ± S.D. = 38.46 ± 30.64 bees; Figure 1(a), while the observations took during the rainy season did not produce a clear peak of activity due to the small number of exiting individuals (Figure 1(b)). Liquid foraging changed in intensity throughout the time of day during the dry season but not in the rainy season where liquid foraging was significantly reduced (dry season: $H_{12} = 195.17$, $P < 0.001$; rainy season: $H_{12} = 104.77$, $P < 0.001$; Figures 3(a) and 3(b)). Foraging for liquid during the dry season began around 6:00 h, with a peak activity at 7:00 h (mean ± S.D = 97.5 ± 12.4 bees) and decreased after 11:00 h. A total of 43,228 bees were observed returning with liquid loads. During the rainy season, the activity of liquid collection showed no significant peak (Figure 2(b)). In this period of observations, 1,959 liquid foragers were recorded.

Collection of pollen, resin, and mud also differed among seasons and time of day (Figures 3(a) and 3(b)). Pollen collection showed a significant variation with relation to the time of day in both seasons (dry season: $H_{12} = 225.26$, $P < 0.001$; rainy season: $H_{12} = 66.65$, $P < 0.001$; Figures 2(a) and 2(b)). 5,198 bees were observed returning with pollen during the dry season and 340 bees during the rainy season. Resin collection peaked at 7:00 h in the dry season and from 8:00 to 10:00 h during the rainy season (dry season: $H_{12} = 80.07$, $P < 0.001$; rainy season: $H_{12} = 32.21$, $P < 0.001$). During the dry and rainy seasons, 6,213 and 118 bees were observed returning with resin, respectively.

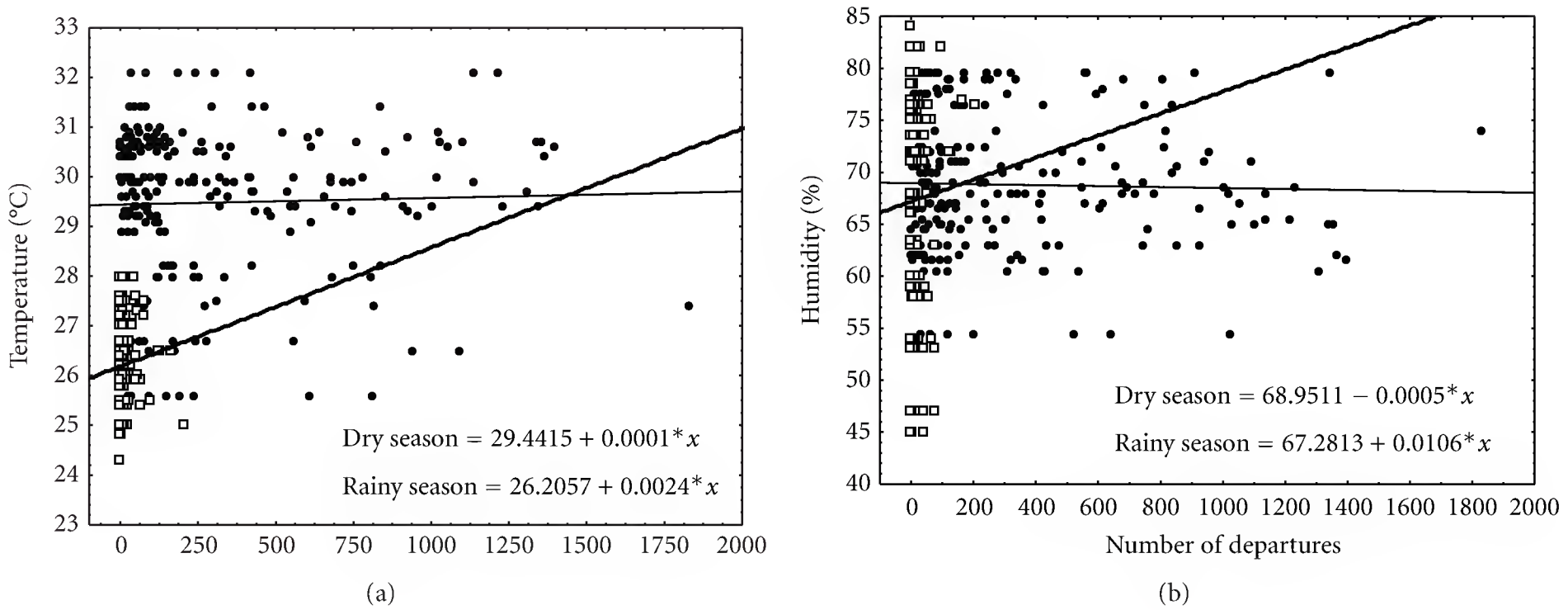

Figure 1: Relationship between temperature (a) and humidity (b) and the number of returning *Melipona asilvai* bees (• dry season; □ rainy season).

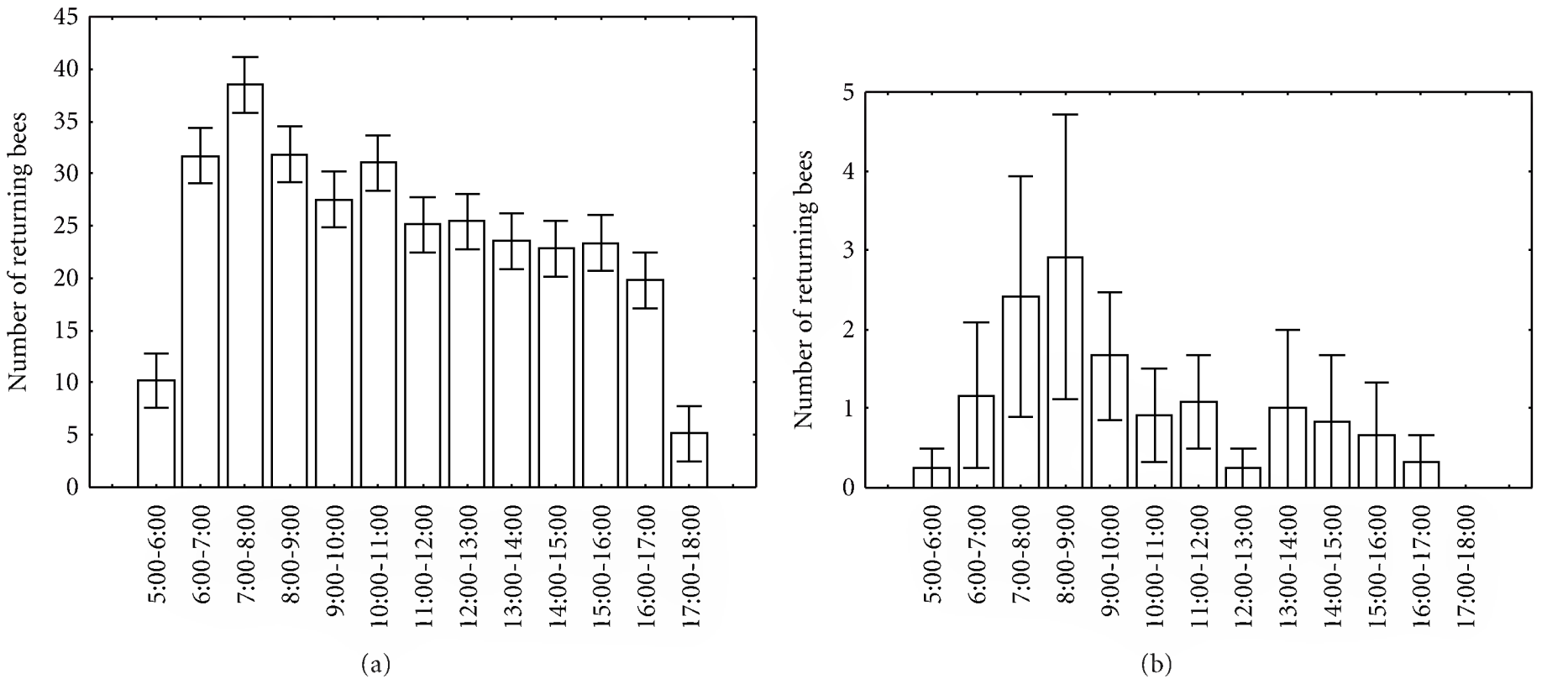

Figure 2: Daily frequency (mean ± SE) of returning *Melipona asilvai* foragers during the 38 days of observations. (a) Dry season and (b) rainy season.

Mud collection was collected throughout the day and exhibited no specific peak activity (Figures 3(a) and 3(b)). This difference was significant for both periods of study (dry season: $H_{12} = 86.19$, $P < 0.001$; rainy season: $H_{12} = 28.68$, $P = 0.004$). 16,106 and 213 returning bees were observed with mud in both seasons, respectively.

3.3. Colony Productivity. The analyses of relative colony productivity showed that all parameters significantly varied between dry and rainy seasons (Figure 4). More nectar pots were observed during the rainy than the dry season (Mann-Whitney U test = 9.10, $P < 0.001$). On the contrary, the number of pollen pots was smaller during dry season (Mann-Whitney U test = 5.15, $P < 0.001$). Brood production nearly suspended during the rainy season, so the number of cells being provisioned was significantly smaller in this season (Mann-Whitney U test = 2.67, $P < 0.05$).

4. Discussion

4.1. Foraging Activities and Seasonality. Our results showed that during the 19 days of study in the rainy season, foraging departures of *M. asilvai* foragers for food resources (liquid and pollen) decreased over 20 times. Collection of resources seems not to be independently influenced by single factors such as temperature or humidity. Another factor that can also affect the foraging activity of stingless bees is the variation in the quantity and quality of food resources between days or seasons [10, 28]. Biesmeijer et al. [9] observed higher concentrations of sugar from nectar

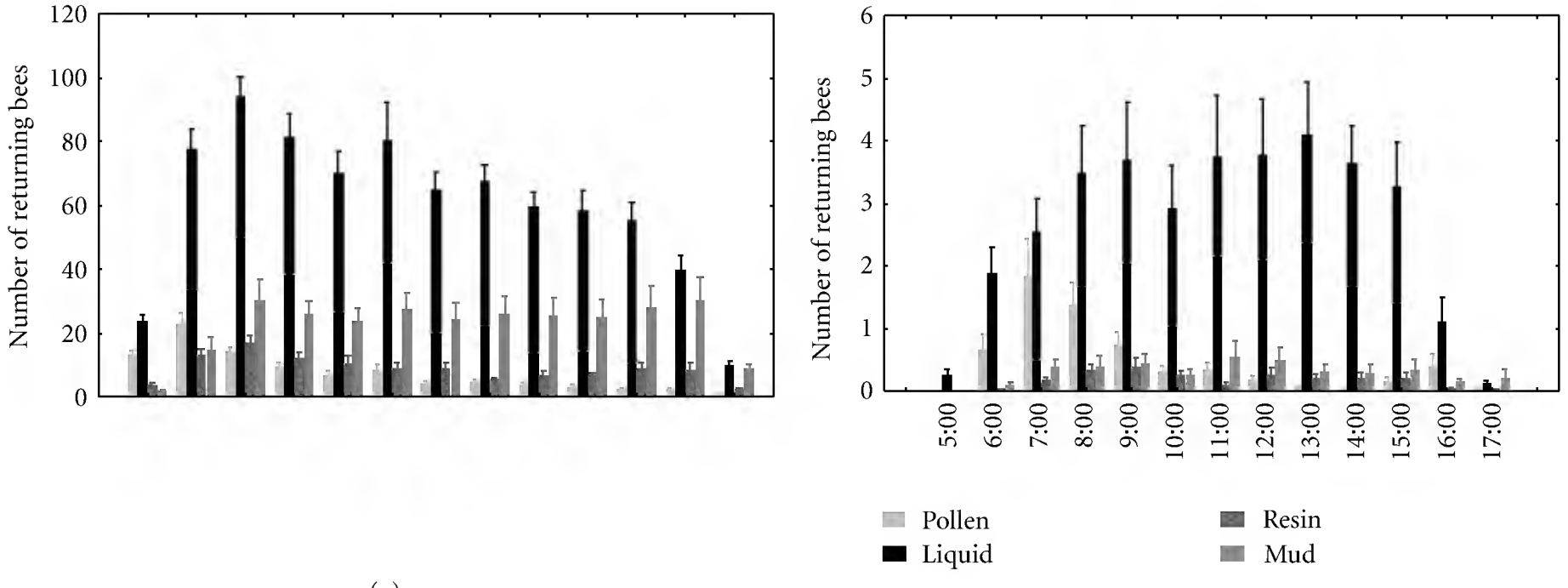

FIGURE 3: Daily frequency (mean ± SD) of returning bees from three *M. asilvai* colonies bringing different types of loads during dry season (a) and rainy season (b).

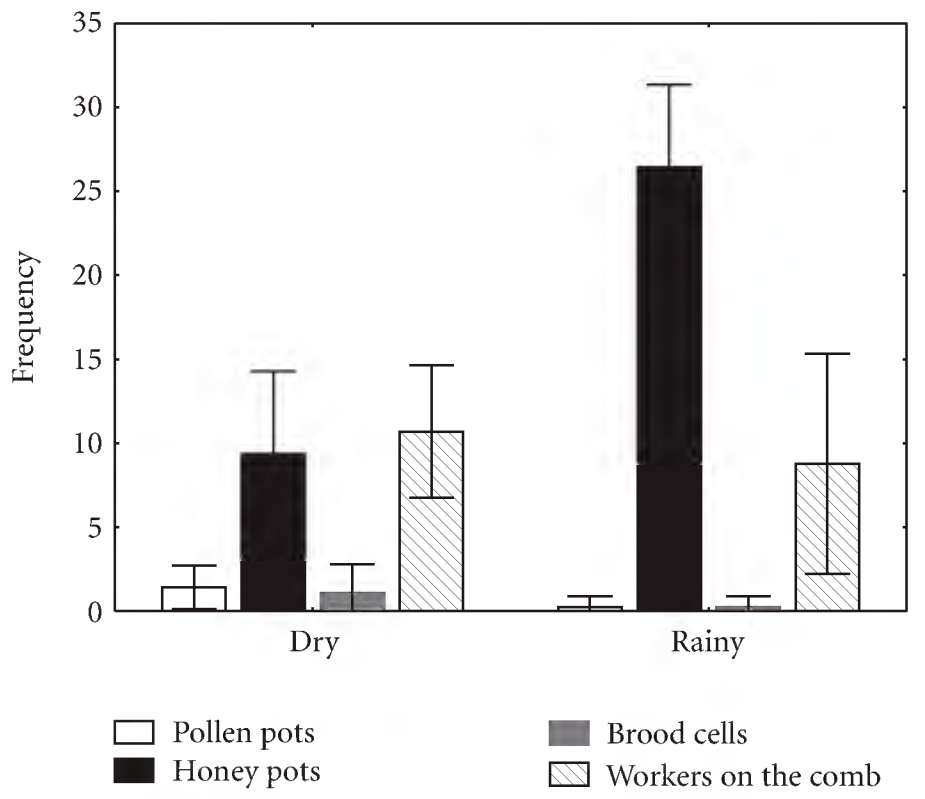

FIGURE 4: Colony productivity (mean ± SD) patterns recorded during dry and rainy seasons.

collected by bees in dryer environments. Indeed, foraging organization is a result of individual foragers responding to environmental changes.

A previous study carried out with the same species in a drier area of Northeast Brazil registered a similar relationship between abiotic factors and foraging activities [29]. Other studies made in higher latitudes verified that temperature and relative humidity are the most limiting factors affecting the peaks of flight activity of stingless bees [17, 21, 23].

4.2. Foraging for Resources, Time of Day, and Season. Our studies showed that pollen collection in *Melipona asilvai* peaked during the first hours of the morning and decreased by the afternoon. This pattern has been seen in other *Melipona* species as well [30, 31]. Hilário et al. [21] observed that in *M. bicolor bicolor* an intense incoming of pollen took place in the early morning, when relative humidity was higher and temperature and light intensity were more moderate. Roubik [10] stated that pollen harvesting in the first hours of day coincides with a higher availability of this resource in the flowers.

Collection of liquids occurred throughout all activity periods in *M. asilvai* colonies. Although a 90% reduction of departures flights had been observed during the rainy season, there was a regular distribution of incoming liquid during the day in both seasons. Pierrot and Schilindwein [31] recorded higher rates of nectar foraging in the afternoon periods for *M. scutellaris*, which could be related to the gradual increase of sugar concentration in insolated flowers [32]. A similar pattern was found in an experiment carried out with *M. rufiventris* in southeast Brazil [24].

Collection of liquid was remarkable during the dry season (see Figure 3(a)). The number of bees returning with liquid loads was around 70% higher than other loads. These results, associated with both a decrease in flight activity and the number of honey pots registered in the rainy season, suggests that *M. asilvai* colonies experience a kind of seasonal diapause. In southern states where seasons are more defined, flight activity of *M. bicolor schencki* and *M. marginata obscurior* was more intense during summer and spring than autumn and winter [33, 34]. Reproductive diapause has been observed in other southern species of stingless bees, such as *Plebeia remota* and *P. droryana* [28, 32].

4.3. Colony Productivity. Season had a significant effect on the relative parameters of colony production in *M. asilvai*. It is known that food resources are critical for the production of workers, queens, and males in stingless bees [35–37]. Although we did not record brood production in this study, it is reasonable to speculate that caste production and colony fission in this species occurs during the dry season when the rhythm of activities is higher.

5. Conclusion

We conclude that the dry-rainy seasonal variation strongly affects external and internal biological parameters of *Melipona asilvai*. Foraging activities decrease by almost 90% from the dry to the rainy seasons, but temperature and humidity were not the main factors influencing departures. Honey storage and a sharp decline in activities during the rainy period suggest a seasonal diapause in this species.

Acknowledgments

This study is part of the M.S. Dissertation of Daniela L. do Nascimento as a requisite of the Post-Graduation Program in Ecology and Conservation (Universidade Federal de Sergipe). The authors thank Hans Kelstrup for his linguistic revision and two anonymous referees for the improvement on the paper. They also thank to Valdson Santos and M.S. José O. Dantas for helping with the management of the colonies and support during the experiments with *M. asilvai*. This study was supported by Fapitec and Capes to D. L. Nascimento and FAPESP (proc. 2010/10027-5) to F. S. Nascimento.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 261316, 9 pages
doi:10.1155/2012/261316

Research Article

Foraging Activity of Native Ants on Trees in Forest Fragments Colonized by the Invasive Ant *Lasius neglectus*

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Received 4 February 2012; Revised 17 April 2012; Accepted 17 April 2012

Academic Editor: James Charles Nieh

Our aim was to investigate the foraging activity of native ants on tree trunks in accordance with their location in forest fragments and the presence or absence of the invasive ant *Lasius neglectus*. Trees were categorized as isolated, edge, or core trees according to their location in forest fragments. In invaded fragments, *Lasius neglectus* had the highest spatial-temporal tree visitation. Isolated trees were visited more and for a longer time by this invasive ant. Invaded fragments had low native ant activity on trees compared to fragments without *L. neglectus*. The few encountered native ant species showed a lower frequency of visitation and for less time in comparison with their spatial-temporal visitation in control fragments. *Crematogaster scutellaris* and *Temnothorax lichtensteini* visited all tree categories in both fragments (invaded or control) but *Lasius grandis* stayed for longer on isolated trees from control fragments. We conclude that in fragments invaded by *Lasius neglectus*, the richness of native ant foraging on trees was negatively affected. Isolated trees close to roads could act as dispersal stepping stones for *Lasius neglectus*.

1. Introduction

In ants, daily and seasonal foraging activity is mainly modulated by the interaction of abiotic and biotic variables [1–4]. Temperature of soil surface and relative humidity has been reported as the most relevant variables that influence ant foraging [5]. However, other abiotic variables such as sunlight, rainfall, wind intensity, atmospheric pressure, and light intensity may influence the activity of some ant species [6–9]. Foraging activity determined by physical variables is modulated by biotic variables such as interspecific competition and habitat structure [3], resource productivity [10], food type, and colony needs [11] and physiological constraints such as heat tolerance [7]. Additionally, the activity of dominant species (*sensu* [12]) may determine the foraging patterns of less dominant species [13]. In this regard, invasive ant species become dominant because of their aggressive behavior and the major abundance that their unicolonial social structure and polygyny (many queens per colony) allow them to achieve in a short time. In consequence, invasive ants monopolize food sources, mainly honeydew-producing insects, negatively affect native arthropods and even small vertebrates, and disrupt and develop mutualisms in native communities [14]. In short, ant-aphid interactions may have strong and pervasive effects extending across multiple trophic levels [15].

The invasive ant *Lasius neglectus* [16] has been proposed by Tsutsui and Suarez [17] as a candidate to become a similar problem to the Argentine ant *Linepithema humile*. Like other invasive ant species, *L. neglectus* relies on honeydew for its main food source and, but for a single instance in a grassland without trees in Tiflis [18], known food sources come exclusively from insect prey and honeydew-producing insects on trees [19]. Thus, here we limit our observations to that particular habitat: trees. *L. neglectus* modifies the arthropod community [20] and does not build elaborate nests. Instead, *L. neglectus* usually nests under flat stones [21], in the topsoil under leaf litter and even

in trash piles (authors pers obs). In human habitats, *L. neglectus* tends to nest inside electrical devices [22]. In Spain, the distribution of *Lasius neglectus* comprises 20 localities. Depending on the locality, its distribution may comprise an area of several hectares where no other ant species are found or there are only a few trees that are eventually shared with other ant species (http://www.creaf.uab.es/xeg/Lasius/Ingles/distribution.htm, last update December 2011). Up to now, this invasive ant species has never been recorded in natural sites in Spain. However, in 2007 several individuals were found foraging in a natural reserve at Argelès-sur-Mer, France (http://www.creaf.uab.es/xeg/Lasius/Ingles/argelessurmer.htm). This highlights the ability of *L. neglectus* to establish in natural areas.

The distribution of invasive ant species is usually shown by placing dots on a map or by painting an entire area [23]. However, a closeup view shows that not all places are equally occupied by an invasive ant species. In this regard, understanding how changes in the spatial-temporal foraging of an invasive species may help to invest control efforts only in hotly invaded areas at the right time. For two years, we have been estimating the attention and abundance of tended aphids for the invasive ant *Lasius neglectus* on oak trees in forest fragments in a suburban area of Catalonia, Spain. Our first impression was that not all trees were equally visited by *L. neglectus* and that some trees are shared or even visited only by native ants. We, therefore, wondered how the spatial-temporal foraging of native ants varies on trees in forest fragments colonized or not by the invasive ant *Lasius neglectus*. During the activity season, we surveyed how many and for how long native ant species foraged on trees in forest fragments colonized or not by this invasive ant. Additionally, we investigated whether the foraging activity of *L. neglectus* varied according to tree location (isolated, edge, or core trees) because invasive ants are mainly associated with disturbed areas as noted by Majer et al. [24] and Suarez et al. [25]. We hypothesized that the native ant species *Lasius neglectus* would occupy more trees located in more disturbed areas (isolated trees) and for a longer time in comparison with native species. Considering the general evidence of the effect of invasive ants on local ants [14], we expected a richer ant community in forest fragments not colonized by *Lasius neglectus*.

2. Material and Methods

2.1. Study Area. This study was performed on the campus of the Autonomous University of Barcelona (41°30′ N, 2°6′ E), an area of 263 ha. Given its biogeographic location, relief and climatic conditions, this area is considered typical Mediterranean mixed holm oak forest. However, this original mixed forest was fragmented due to the agricultural and forest activities performed over the last two centuries. In the late sixties, when the university was built, the campus area was covered by 51.4 ha of fragmented forest [26]. At that time, in Catalonia, land use changed due to the abandonment of agricultural activities and the replacement of firewood with new sources of energy. In consequence, the forest recovered and nowadays 81 ha of the campus is fragmented into the original holm oak (*Quercus ilex* L.) forest, mixed forest (*Pinus* spp. plus *Quercus* spp.), and pine forest (*Pinus halepensis* Mill. or *Pinus pinea* L.). In the first two forest categories, the understory comprises *Asparagus acutifolius* L., *Crataegus monogyna* Jacq., *Rubia peregrina* L., *Rubus ulmifolius* Schott, *Ruscus aculeatus* L., *Smilax aspera* L., *Viburnum tinus* L. and *Hedera helix* L, and in more open forest areas *Spartium junceum* L., *Juniperus communis* L, and *Rosmarinus officinalis* L. In pine forest, the understory is scarce, with *Brachypodium sylvaticum* (Huds.) Beauv. and *Ulex parviflorus* Pourr.

The climate is Mediterranean, with a wet spring and fall and a dry winter and summer. Mean annual temperature is 16.5°C and mean annual rainfall is 575 mm.

In 1997, *Lasius neglectus* was first recorded in a pile of rubble close to one of the University's railway stations. Nowadays, this ant occupies 15% of the campus area including forests, shrubland, gardens, and pavements (Figure 1).

2.2. Forest Fragment Traits and Surveys. We chose as large an area as possible within different fragments of mixed forest in order to survey all trees. We were constrained by the presence of dense understory mainly composed of *Smilax aspera* and *Rubia peregrina* and *Rubus ulmifolius* and by ravines. In April 2005, we chose three areas of 0.14 ha, 0.032 ha, and 0.103 ha occupied almost exclusively by *Lasius neglectus* (Figure 1). In previous years, we noticed that in invaded fragments some trees were regularly visited by native ant species. These invaded areas were separated by roads (distance range: 80–220 m). Two of the chosen areas border grassland (0.094 ha and 0.248 ha) where there were isolated trees. In forest sites, tree density varied between 364 and 844 trees/ha. Meanwhile on grassland, tree density was 46–53 trees/ha.

In April 2006, we added to the study four areas of forest fragments of 0.12 ha, 0.084 ha, 0.04 ha, and 0.057 ha that were not occupied by *Lasius neglectus*. The distance between them was 220 to 2600 m while the distance from forest fragments invaded by *Lasius neglectus* ranged from 720 to 2370 m. Tree density was 298–575 tree/ha.

In all forest fragments, holm oaks represented 20–94% of the surveyed trees. The other tree species included in the fragments were *Quercus humilis* (20–38%), *Pinus halepensis* (20–60%), and *Populus alba* (17–31%).

We measured tree diameters at breast height (DBH) and differentiated trees according to their location in the forest. We considered three categories of tree: isolated trees (I) when the tree trunk was located more than 5 m from the forest and their crown did not contact the forest canopy, edge trees (E) when they bordered fields or roads, and finally, core trees (C) when the trunk was located 5 m from the forest edge and more than 60% of their crown was in contact with the crown of other closer trees. Isolated trees close to invaded forest were considered part of the invaded area.

In this study, on each sampling date, we considered a tree to be visited by a given ant species when we saw a trail on the tree trunk with workers moving downwards with their gasters full of honeydew or a few workers climbing to

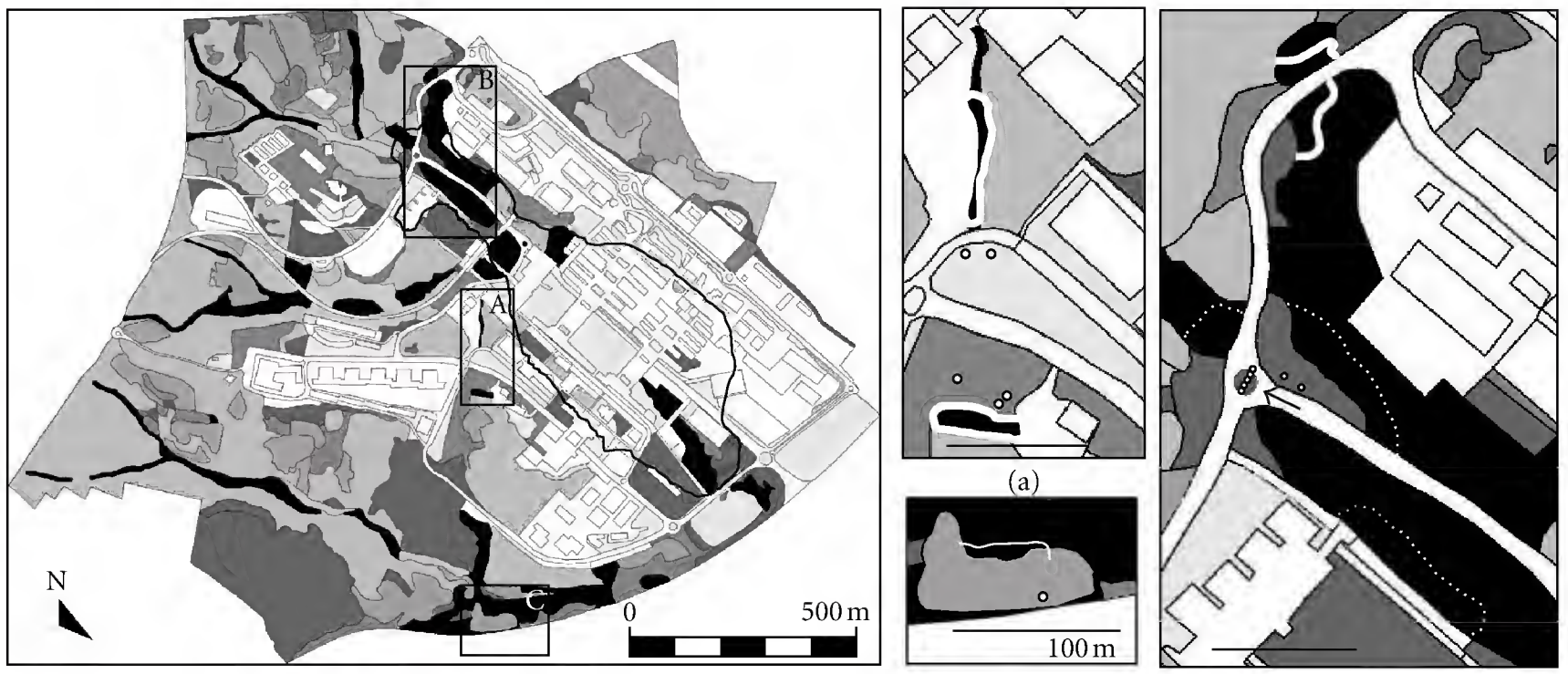

FIGURE 1: The University campus is composed by three main units. The first unit comprises all the university and transportation infrastructure like buildings, parkings, railway stations, roads, and paths. The agroforestal unit is composed by natural areas such as mixed and pine forests, shrubland, grassland and reedbed. Finally, the gardened unit included grass areas with several isolated trees and bushes. The area of the campus invaded by *L. neglectus* is surrounded by a black line. On the right side, areas (a, b, and c) of the chosen invaded (dotted line) and control (continuous line) forest fragments have been enlarged. Isolated trees are shown with small white circles. The arrow, in figure (b) points a roundabout where there are five isolated trees colonized by *L. neglectus*.

explore the crown. Between late April and mid October, we recorded all ant species that were observed climbing all tree trunks, comprising in total 120 trees in invaded fragments and 78 trees in control fragments. We identified ant species foraging on tree trunks in the field, when possible, or we took samples for identification in the laboratory. Trees were observed between 9 h and 13 h (solar time) every 25 ± 2 days, (mean ± SE). Invaded fragments were surveyed in both years (2005 and 2006), while control fragments were surveyed in one year (2006).

2.3. Statistical Analysis. The size and shape of the chosen areas of fragment types (invaded or not) were compared using a t-test. Tree abundance of each tree category (isolated, edge and core trees) and tree diameter were compared separately using a two factor ANOVA. We considered fragment type and tree category as factors.

The analysis of foraging activity was divided into spatial tree visitation, that is, how many trees of each category were visited and temporal tree visitation, that is, for how long trees of each category were visited. We expressed foraging activity as a percentage of visited trees from each tree category and fragment type. For example, in invaded fragment #number 2, there were 14 core trees. In May, 11 trees were visited by the invasive ant *L. neglectus*. So, the tree visitation score was 78.5% (11/14). Prior to analysis, percentages were subjected to the arcsin transformation although raw data are presented in the text.

2.4. Foraging Activity and Richness of Native Ants. We compared ant species richness using a t-test considering fragment type (invaded or control) as the grouping variable.

Spatial tree visitation by native ants was compared using two-way ANOVA repeated measures including fragment type, tree category, and date of survey as fixed factors and the percentage of visited trees as the dependent variable. Temporal tree occupancy was compared using a two way ANOVA considering fragment type and tree category, as fixed factors and the number of months that a given tree was visited by native ants as the dependent variable.

When significant differences were found ($P < 0.05$) Tukey post hoc comparisons were run. All analyses were performed using Statistica 6.0 [27].

2.5. Foraging Activity of L. neglectus and Its Effect on Native Ants. In invaded forest fragments, spatial tree visitation of *L. neglectus* was analyzed using two-way repeated measures ANOVA including ant type (invasive or native), tree category (isolated, edge, core trees), and date of survey (repeated measure) as fixed factors and the percentage of visited trees as the dependent variable. In this study, we will report only those results related to the main effect of the factors or only their interaction, because at this stage we are not specifically interested in seasonal patterns. Temporal tree occupancy was compared using a two-way ANOVA considering ant type and tree category as fixed factors and the number of months

Table 1: Mean (SE) of forest fragment size, tree abundance and tree diameters at fragments occupied by the invasive ant *Lasius neglectus* (LN) or native ants (NA). Trees were categorized as: isolated trees (I), edge trees (E), or core trees (C). Total abundance of each category is after (SE). Different letters showed significant differences of post hoc comparisons of the interaction between tree category x fragment type (Tukey, $P < 0.05$).

Fragment type	Area (ha)	Edge (m)	Abundance			Diameter (cm)		
			I	E	C	I	E	C
LN	0.092 (0.031)	81.67 (6.74)	2.33 c (1.45) 7	14.67 ab (2.33) 44	23.0 a (4.51) 69	29.49 (2.78)	31.58 (4.00)	28.47 (2.57)
NA	0.075 (0.017)	53.30 (23.98)	1.75 c (0.85) 7	12.75 ab (3.66) 51	5.0 bc (1.08) 20	25.75 (5.12)	28.62 (2.13)	21.51 (2.68)

that a given tree was visited by invasive or native ants as the dependent variable.

3. Results

3.1. Forest Fragment Traits. Both fragment types (invaded or control) had similar size and shape characteristics (area: $t = 0.49$, $df = 5$, $P = 0.646$; edge: $t = 0.88$, $df = 5$, $P = 0.421$; edge/area: $t = 1.26$, $df = 5$, $P = 0.264$, Table 1). The interaction between tree category and fragment type was significant (ANOVA, fragment type x category interaction, $F_{2,15} = 5.74$, $P = 0.014$, Table 1). This was due to less abundance of isolated trees in both forest types (Tukey, $P < 0.05$). On the contrary, core trees were significantly more abundant in invaded fragments (Tukey, $P < 0.05$). However, edge trees did not differ between forest types (Tukey, $P > 0.05$). Tree diameters were similar for both fragment types, (ANOVA, $F_{2,192} = 1.14$, $P = 0.320$) and for all categories (ANOVA, $F_{2,192} = 0.79$, $P = 0.455$, Table 1). Given the general similarity of those characteristics and the common origin of the forest fragment, we assumed that possible differences in ant foraging in trees in invaded and noninvaded fragments are attributable to the presence of the invader, and not to any environmental gradient.

3.2. Foraging Activity and Richness of Native Ants. Richness of native ants foraging on trees was significantly higher in control than in invaded fragments (2006, $t = -6.35$, $df = 5$, $P = 0.0014$, invaded: 6.67 ± 0.31, control: 9.25 ± 0.27) (Table 2). Relative frequencies of native ants diminished markedly in invaded fragments (Table 2).

The spatial foraging of native ants in both fragment types (invaded or control) was the same for all tree categories (repeated measures ANOVA, interaction of forest type x tree category, $F_{2,13} = 2.08$, $P = 0.164$, Figure 2(a)) but in control forest fragments they foraged significantly more than in invaded fragments (repeated measures ANOVA, $F_{1,13} = 43.28$, $P < 0.001$, control: 57.63 ± 3.88% visited trees, invaded: 13.75 ± 4.59% visited trees).

Native ants, in control fragments, remained in all tree categories for a similar time but in invaded fragments they remained significantly more on edge than core trees but some isolated trees were eventually visited (ANOVA, $F_{2,192} = 4.06$, $P = 0.019$, Tukey $P < 0.05$, Figure 2(b)).

Invaded and control forest fragments shared three native ant species whose frequency enabled statistical analysis of their spatial-temporal foraging on trees depending on the fragment type (invaded or control). They are *Lasius grandis*, *Crematogaster scutellaris*, and *Temnothorax lichtensteini* (Table 2).

Spatial tree foraging of *Crematogaster scutellaris* (Cs) and *Temnothorax lichtensteini* (Tl) was similar for both forest fragments (repeated ANOVA measures, Cs, $F_{1,13} = 0.93$, $P = 0.352$; Tl: $F_{1,13} = 0.22$, $P = 0.647$). The interactions between fragment type and tree category were not significant (repeated ANOVA measures, Cs, $F_{2,13} = 0.41$, $P = 0.672$; Tl: $F_{2,13} = 1.04$, $P = 0.382$). In invaded fragments, *Lasius grandis* appeared in only one fragment, so it was not possible to analyze its spatial tree visitation.

Temporal tree foraging of *Crematogaster scutellaris* and *Temnothorax lichtensteini* was similar in both forest fragments (ANOVA, Cs, $F_{1,192} = 0.78$, $P = 0.379$; Tl: $F_{1,192} = 0.58$, $P = 0.446$) but the permanence of *Lasius grandis* differed between fragment types (ANOVA, $F_{1,192} = 64.37$, $P < 0.001$). Post hoc comparisons showed that *Lasius grandis* in control fragments remained for significantly ($P < 0.05$) more months on isolated trees (2.43 ± 0.30 months) than on edge or core trees (edge: 1.22 ± 0.11 months, core: 0.65 ± 0.18 months) while in invaded fragments it remained for a similar time ($P > 0.05$) on edge (1.36 ± 0.12 months) and core trees (0.03 ± 0.1 months). There were no isolated trees in the only invaded fragment where *Lasius grandis* was found.

3.3. Foraging Activity of L. neglectus and Its Effect on Native Ants. In both years, the invasive ant *Lasius neglectus* (LN) visited significantly more trees than native ants (NA) (repeated ANOVA measures, mean ± SE, year 2005, LN: 35.67 ± 3.11% visited trees, NA: 7.71 ± 3.11% visited trees; year 2006, LN: 29.21 ± 3.87% visited trees, NA: 13.75 ± 3.87% visited trees, Table 3). The interaction between ant type and tree category (isolated, edge, or core trees) was significant in both years (Table 3). Post hoc comparisons showed that in both years the invasive ant foraged significantly more on isolated trees than core trees (Tukey, $P < 0.05$) but edge tree visitation did not differ significantly from the other two categories (Tukey, $P > 0.05$, year 2005, isolated trees: 54.36 ± 6.10%, edge trees: 33.75 ± 4.98%, core trees: 18.91 ± 4.98%; year 2006, Figure 3(a)). Native ants in 2005 only visited edge (13.26 ± 4.98%) and core trees (9.86 ± 4.98%, $P > 0.05$) while in 2006 all tree categories were visited in similar percentages (Figure 3(a)).

In both years, *Lasius neglectus* remained on a given tree for significantly more months (year 2005: 2.78 ± 0.19 months; 2006: 2.15 ± 0.16 months) compared with native ants (year 2005: 0.60 ± 0.19 months; 2006: 0.98 ± 0.16

Table 2: Relative frequency (absolute frequency/number of observations) of tree visitation by each ant species at invaded (I) or control (C) forest during the activity period (7 months; in 2006). In brackets are shown absolute frequency that is the number of times each ant species was found along the seven censuses (number of observations: 840 at invaded fragments, 546 at control fragments). Ants were discriminated according to their nesting site: soil (S), arboricolous (A), or arboricolous-under bark (U).

Ant specie	I	C	Nesting site
Lasius neglectus	0.173 (145)		S
Lasius grandis	0.021 (18)	0.176 (96)	S
Lasius emarginatus	0.001 (1)	0.101 (55)	S
Crematogaster scutellaris	0.095 (84)	0.148 (80)	A
Camponotus aethiops		0.064 (35)	S
Camponotus cruentatus	0.008 (7)	0.035 (19)	S
Camponotus piceus		0.002 (1)	S
Camponotus truncatus	0.005 (4)	0.060 (33)	A
Formica rufibarbis		0.002 (1)	S
Myrmica spinosior		0.015 (8)	S
Pheidole pallidula	0.001 (1)	0.002 (1)	S
Plagiolepis pygmaea	0.004 (3)	0.031 (17)	S
Temnothorax lichtensteini	0.026 (22)	0.038 (21)	U
Native ant species richness	8	12	

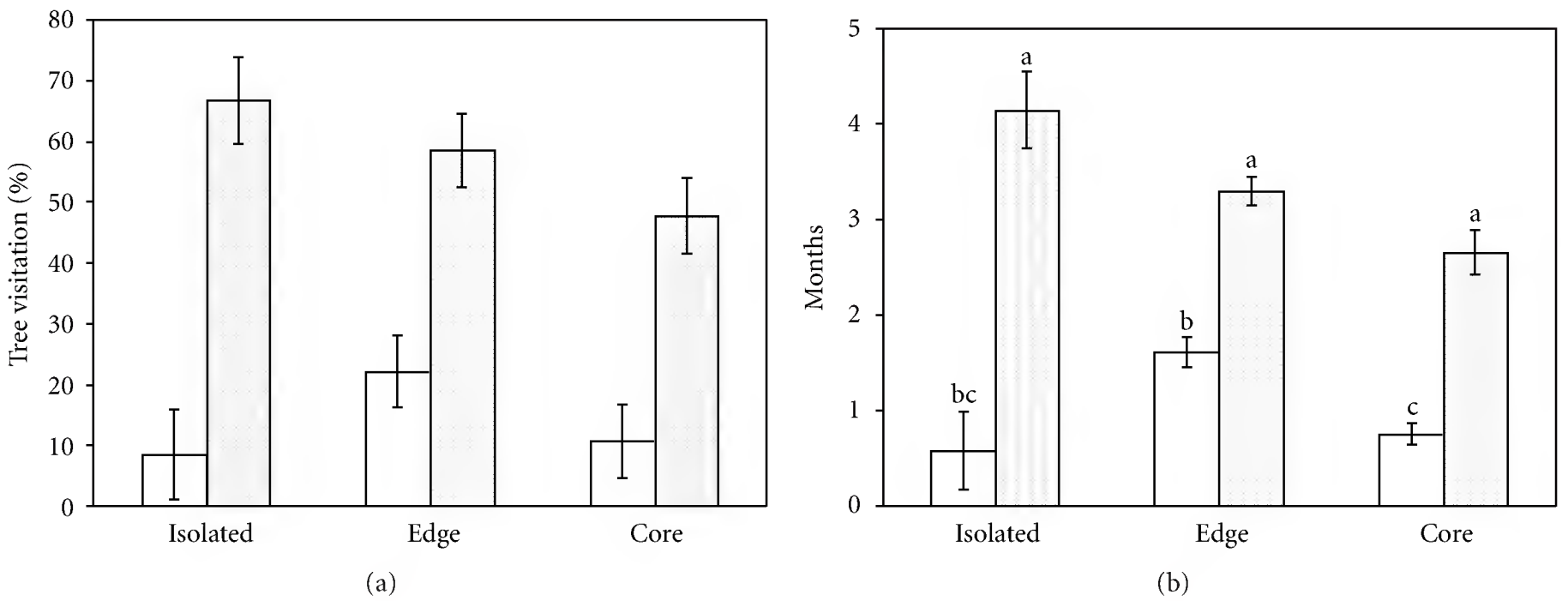

Figure 2: Foraging activity of native ants on trees is shown as the percentage (mean ± SE) of visited trees (a) and the months they remain visited (b) per tree category in 2006 at invaded fragments (bars in white) or at control fragments (dotted bars). Different letters showed statistical differences of the tree category x ant type interaction ($P < 0.05$).

months, ANOVA, year 2005: $F_{1,232} = 66.29$, $P < 0.001$; year 2006: $F_{1,234} = 20.67$, $P < 0.001$). The interaction between ant type and tree category was significant in both years (ANOVA, year 2005: $F_{2,232} = 15.27$, $P < 0.001$; year 2006: $F_{2,234} = 15.45$, $P < 0.001$). In both years, the invasive ant remained on isolated trees for significantly more months (year 2005: 4.67 ± 0.50 months) in comparison to the permanence on the other two tree categories ($P < 0.05$, year 2006, Figure 3(b)). The permanence on edge or core trees of the invasive ant differed significantly ($P < 0.05$) only in 2005 (year 2005, edge: 2.41 ± 0.19 months, core: 1.26 ± 0.15 months; year 2006, Figure 3(b)). Native ants in 2005, a year in which they did not visit isolated trees, remained for the same time on edge (1.02 ± 0.19 months) and core trees (0.77 ± 0.15 months, $P > 0.05$), whereas in 2006 the permanence of native ants was higher on edge trees ($P < 0.05$, Figure 3(b)).

4. Discussion

The consequences of ant invasions on native ant biodiversity has been widely explored. The invasive Argentine ant, *Linepithema humile*, has competitively displaced native ant species as it has spread in its introduced range [28, 29]. Similarly, the red imported fire ant, *Solenopsis invicta*, devastated native fauna as it expanded its range across the southeastern United States [30]. In monsoonal Australia, high abundance of the big-headed ant, *Pheidole megacephala*, corresponded with a 42–85% decrease in the abundance

Table 3: Repeated measures ANOVA of tree visitation (%) at invaded fragments, depending on ant type (invasive or native) and tree category (isolated, edge or core trees). Significant effects ($P < 0.05$) are shown in bold.

	2005			2006		
Source effect	*df*	*F*	*P*	*df*	*F*	*P*
Ant type	1	36.72	**<0.0001**	1	7.43	**0.021**
Tree category	2	1.17	0.349	2	2.79	0.108
Ant type x Tree category	2	9.67	**0.005**	2	7.48	**0.010**

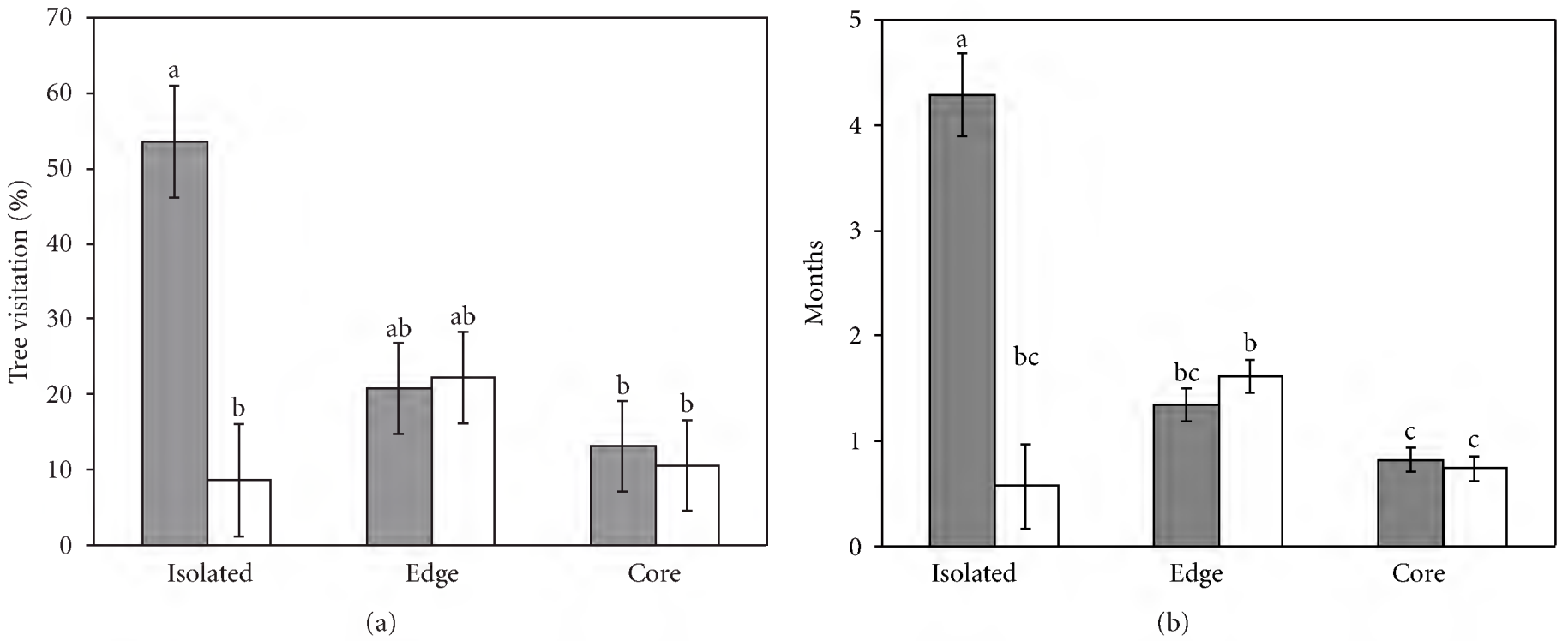

Figure 3: Foraging activity at invaded forest fragments is shown as the percentage (mean ± SE) of visited trees (a) and the months they remain visited (b) per tree category in 2006. Bars showing trees visited by *Lasius neglectus* are in grey and visited by native ants are shown in white. Different letters showed statistical differences of post hoc comparisons (tree category x ant type effect) ($P < 0.05$).

of other native invertebrates [31], and the yellow crazy ant, *Anoplolepis gracilipes,* displaced other ant species as its activity increased and boundaries expanded on Christmas Island [32]. According to Andersen [33], only those native ant species with specialist foraging times or microhabitat preferences are the most resistant to elimination.

4.1. Foraging Activity and Richness of Native Ants. In our study site, the richness of ants foraging in trees in invaded forest fragments was significantly lower in comparison with control forest fragments. The strongest effect of the presence of *L. neglectus* is for the congeneric *L. grandis* and the weakest effect is for smaller and cryptic species. This general finding was already reported by Ward [34] in an Argentine ant invasion in natural habitats of the lower Sacramento River Valley. This effect of *L. neglectus* on the ant community has been reported by other authors at sites with a higher density of this invasive ant [18, 35]. The native ants *Lasius grandis*, *Crematogaster scutellaris,* and *Temnothorax lichtensteini,* were encountered in both fragment types (control or invaded) (Table 3) but showed a lower frequency in invaded fragments. All these native species collect honeydew and small insects. In the invaded fragments, the few trees on which *Crematogaster scutellaris* and *Temnothorax lichtensteini* were able to forage were visited with the same spatial-temporal tree visitation as in the control fragments. *Lasius grandis* was found in only one invaded fragment so comparison was not possible with the situation in control fragments where it remained for more months on isolated trees at the highest tree visitation frequency. This could be the consequence of its nesting habit. The native ant *Lasius grandis* is able to nest in open areas that are associated with isolated trees and dig burrows at the base of visited trees from where workers climb to tend aphids (Paris pers. observ.). The other two native ant species that appeared in all fragments, *Crematogaster scutellaris* and *Temnothorax lichtensteini*, have their own ecological particularities that may enable them to coexist with *L. neglectus*. In mixed forests, *Crematogaster scutellaris* is considered a dominant ant species or codominant with *Pheidolle pallidula* [36, 37]. This native ant is an arboricolous polydomous nesting ant that changes its nesting location frequently and is highly aggressive. These traits may enable *Crematogaster scutellaris* to coexist with *Lasius neglectus* in invaded fragments. Marlier et al. [36] observed, in a fig plantation (*Ficus carica* L.) with the presence of *Lasius neglectus*, that the presence of *Crematogaster scutellaris* did not influence invasive ant activity. Instead, the opposite effect is probably certain although this should be specifically tested. The frequency of tree visitation suggests that in invaded forest fragments *Lasius neglectus* negatively affected the presence of *Crematogaster scutellaris*. In fact, in Doñana National Park, *Crematogaster scutellaris* colonies were successfully displaced from cork oak trees by another invasive ant: *Linepithema humile* [37]. The other native ant species found in all invaded fragments, *Temnothorax lichtensteini*, is a cryptic

species that nests under bark and its small size and low abundance likely diminished the probability of encountering *Lasius neglectus*. Other authors have also reported that some native ant species are able to coexist with invasive ants. On Christmas Island, *Paratrechina minutula* and *Paratrechina longicornis* were commonly found in the same area as the invasive ant *Anoplolepis gracilipes* supercolony [32]. In Japanese urban parks, *Paratrechina sakurae* and *Camponotus vitiosus* coexisted with the invasive ant *Linepithema humile* [38].

Although our monthly surveys were conducted between 9 and 13 PM, we do not expect the situation for native species found in invaded fragments to change over the course of the day because of their foraging patterns. Previous data on *Lasius neglectus* activity showed that this invasive ant has a 24 hr activity cycle (http://www.creaf.uab.es/xeg/Lasius/Ingles/gr2dailyactivity.htm). Concerning *Lasius grandis*, we do not have a detailed 24 hr activity cycle and no information was found in the literature. But a survey performed in the invaded fragments at 6 hour intervals in previous years showed that *L. grandis* was active all day. In fact, other *Lasius* (s.str.) ant species also showed a 24 hr activity period. *Lasius lasioides* in northern Tuscany, Italy (Figure 1 [39]), showed continuous activity between May and July. In Maryland, USA *Lasius alienus* in a woodlot of a second-growth forest composed mostly of oaks (*Quercus* spp.), and Virginia pines (*Pinus virginianus*) also showed an activity period of 24 hrs (Figure 3 [40]). According to Redolfi et al. [41], the maximum foraging pattern of *Crematogaster scutellaris* occurs mainly between 9AM and 16 PM. Concerning *T. lichtensteini*, which is one of the most abundant ant species in Catalonian forests [42], we found no information about its daily foraging pattern. However, considering that this ant species nests under the bark, that its nest comprises less than 200 individuals, and also that is a timid ant species that forages alone and avoids competition, its presence was probably not perceived by *L. neglectus*.

Some field observations lead us to speculate on how *Lasius neglectus* may displace native ants. First, the abundance of *Lasius neglectus* in trees and soil is higher compared with native ants [35]. This higher abundance increases the possibility of finding and monopolizing food resources to the detriment of native ants [12]. Second, on tree trunk trails, when *Lasius neglectus* workers find a native ant worker, they try to capture it or show highly aggressive behavior towards it by pulling their legs or antennae. This behavior should disrupt native ant foraging on the canopy, diminishing the food supply for native colonies. The aggressive behavior of *Lasius neglectus* towards native ants has recently been observed in laboratory aggression tests with *Lasius neglectus* and other *Lasius* native ants: attacks by *Lasius neglectus* were performed faster and most frequently against *Lasius grandis*, were intermediate against *Lasius emarginatus*, and delayed in time and less frequent against *Lasius cinereus* [43]. Finally, recently fertilized native queens of *Messor* sp and *Lasius grandis* that landed on invaded forest fragments were captured immediately by *Lasius neglectus* workers (Paris pers. observ.). Hence, the invasive ant may directly interfere with the establishment of new native colonies.

4.2. Foraging Activity of L. neglectus and Its Effect on Native Ants. In both years, spatial-temporal tree visitation by the invasive ant *Lasius neglectus* was higher than that of the native ants found in invaded fragments. In particular, isolated trees were more visited and for a longer time by the invasive ant in comparison with other tree categories and with native ant foraging on trees. The polydomous colony structure of *L. neglectus* enables them to move freely among trees with a higher aphid abundance. On the contrary, the native ants may deal with territorial constraints that inhibit them from foraging for a long time on trees previously occupied by other native ants. The Argentine ant *Linepithema humile* is also prone to relocate its nest close to food sources on trees and move away when sources are exhausted or workers do not have access to climb the tree [44]. This strategy enables invasive ants to monopolize honeydew sources in order to maintain the large worker activity for community dominance. On the other hand, the fragmentation of the forest also plays an important role in modifying, at the edges, the availability of honeydew sources, the environment, and the ant community. These factors may interact to favor the foraging of *L. neglectus* and some native ants in the case of control fragments. In fact, several studies have recorded increased abundance of tended phytophagous insects such as aphids and treehoppers, on isolated and edge trees from patches of scrubland, neotropical savanna and tropical and temperate forest fragments [45–47]. This edge effect appears to be the result of the interaction between two adjacent ecosystems when they are separated by an abrupt limit [48]. The response may differ depending on the group. In rainforests, some insect groups respond positively to edges while others are negatively affected. Certain termites, leafhoppers, scale insects, aphids, aphid-tending ants [46], and light-loving butterflies [49] increase near edges. In particular, ant-tended aphids increase on isolated trees [47]. On the contrary, numerous bees, wasps [46], ants and butterflies [49] respond negatively to edges. Additionally, at forest edges, ant richness diminishes, ant community composition is modified [50, 51], and a variety of ecosystem processes [52], such as seed dispersion by ants, may change [53]. In a previous study in the same area, we found no differences in aphid abundance tended by *L. neglectus* or the native ant *L. grandis* on holm oaks located at the edge and isolated [19]. However, not all trees were surveyed due to their height and the slope of the area. Therefore, we cannot discount the possibility of there being a gradient in the abundance of tended-aphids from the core of the forest fragment to the edges.

5. Conclusions

In invaded fragments, spatial-temporal foraging of native ants and their richness on trees was strongly diminished in comparison with control fragments. However, the native ants *Crematogaster scutellaris* and *Temnothorax lichtensteini*, both arboricolous, were able to coexist with the invasive ant but showed a lower frequency of foraging on trees and remained for less time in comparison with their permanence in control fragments. The mechanisms that may enable coexistence

between native and *L. neglectus* were a combination of small body size, arboreal nesting habits, and cryptic behavior. Additional sampling approaches (pitfall trap captures, presence in baits, leaf litter sampling) are needed to ascertain the generality of those mechanisms. Some uncoupling of ground foraging and tree foraging levels has been detected in Argentine ants [54].

Forest fragments with high edge-to-interior ratios or disturbance-induced edges are highly susceptible to ant invasion, which can reach natural areas using roads and forest edges as dispersion paths [14, 25, 26]. Isolated trees are usually found on paths and roadsides and have been proposed as spreading corridors for *Lasius neglectus* [55]. In fact, isolated trees were visited more and for a longer time by *Lasius neglectus* than by native ants. Preventing the abundance of aphids on isolated trees or making it difficult for ants to climb trunks should help prevent this invasive ant from reaching other sites. Additionally, monitoring of road edges that pass through an invaded area will help with the early detection of new propagules of *L. neglectus*.

Acknowledgments

The comments of two reviewers added value to the manuscript and pointed to relevant literature. C. Paris would like to thank the Government of Catalonia for the grant for young researchers (2003FI 00342) and to Elizabeth Padilla for helping in field work. Funding was provided by MEC/FEDER CGL 2004-05240-CO2/01 and CGL2007-64080-C02-01.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 423756, 6 pages
doi:10.1155/2012/423756

Research Article

Insights into Population Origins of Neotropical *Junonia* (Lepidoptera: Nymphalidae: Nymphalinae) Based on Mitochondrial DNA

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Received 22 February 2012; Revised 26 April 2012; Accepted 3 May 2012

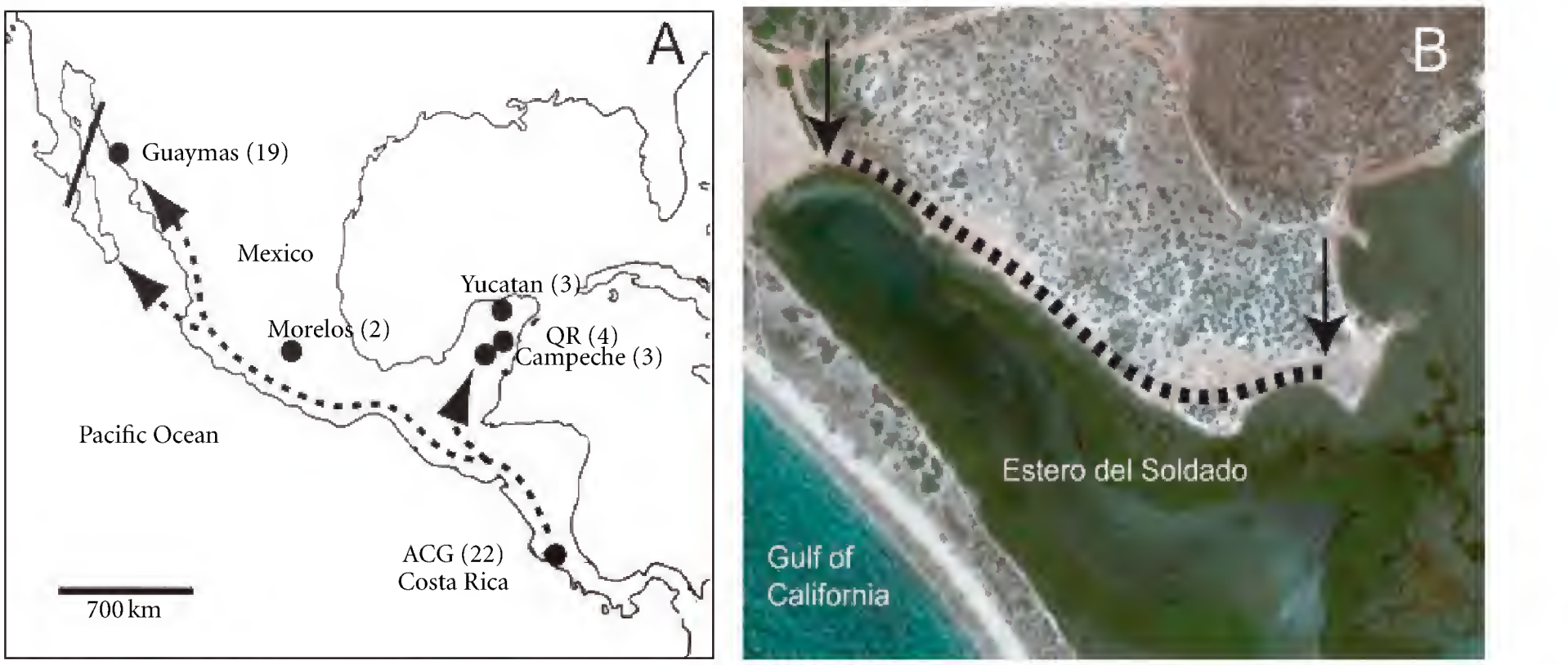
Academic Editor: David Roubik

Cytochrome *c* oxidase subunit I (COI) sequences were used to estimate demographic histories of populations of the buckeye butterfly *Junonia genoveva* (Cramer) from Costa Rica and Mexico. Previous studies have revealed significant structure between populations of *J. genoveva* from coastal regions of northwestern Mexico, which utilize black mangrove *Avicennia germinans* (Acanthaceae) as a larval host plant, and inland populations from Costa Rica that feed on different hosts in the families Acanthaceae and Verbenaceae. The Mexico population of *J. genoveva* reported on here is located near the Northern limit of black mangrove habitat on the Pacific coast of North America and is hypothesized to have been established by northward migrations and colonization from southern source populations. The mismatch distribution, Bayesian skyline analyses, and maximum likelihood analyses carried out in FLUCTUATE were used to estimate changes in female effective population size (N_{ef}) over time in the two populations. Differences found in COI haplotype diversity, present-day N_{ef}, and the timing of population expansions are consistent with the hypothesis that the Mexico population of *J. genoveva* is the more recently evolved.

1. Introduction

The genus *Junonia* Hübner comprises approximately 30+ species of butterflies commonly known as buckeyes and pansies which are distributed worldwide, predominately in the tropics. The New World fauna is thought to have originated about 2–4 million years ago (Ma) from colonizing individuals from Africa or Asia [1]. The number of species of *Junonia* in the New World is uncertain, but at least three, including *J. evarete* (Cramer), *J. genoveva* (Cramer), and *J. coenia* Hübner, are reported for North America [2]. *Junonia evarete* and *J. genoveva* are also found in South America and the Caribbean.

Molecular studies using both mitochondrial and nuclear DNA suggest that two distinct genetic lineages (clades) of *Junonia* began to differentiate in the New World shortly after colonization [1, 3]. Evidence for the relatively recent speciation, together with the pronounced intraspecific phenotypic variability and tendency to hybridize [4], has caused much taxonomic confusion within the genus, especially in distinguishing *J. evarete* and *J. genoveva* [5]. The populations of *Junonia* treated here typically have been assigned to *J. evarete* [6–8], but because these populations are genetically distinct from *J. evarete*, it has been suggested that they be provisionally reassigned to *J. genoveva* [3]. Significant structure was found between a coastal population of *J. genoveva* from northwestern Mexico and an inland population from Costa Rica ($\Phi_{ST} = 0.398$) [3], possibly owing to the large geographic separation (approximately 3250 km; Figure 1(a)) and different host plant preferences. The northwest Mexico population utilizes black mangrove, *Avicennia germinans* (L.) L. (Acanthaceae), as a larval host [9], whereas the Costa Rica population feeds on *Dyschoriste valeriana* Leonard (Acanthaceae) and *Stachytarpheta jamaicensis* (L.) Vahl (Verbenaceae) [10]. The higher COI haplotype and nucleotide diversities found in the Costa Rica population compared with the Mexico population suggested a scenario in which dispersal and colonization of *J. genoveva* proceeded



(a) (b)

FIGURE 1: (a) Map showing location of sampling sites for *Junonia genoveva* at Estero del Soldado, near Guaymas, Sonora, Mexico and at the Area de Conservación Guanacaste (ACG) in Costa Rica. The diagonal line near Guaymas represents the northern distribution limit of black mangrove in the littoral of western North America [11]. Additional GenBank records for *J. genoveva* from the states of Morelos, Campeche, Quintana Roo (QR), and Yucatán in southern Mexico are also shown. The numbers of individuals from each locality are given in parentheses. Arrows show hypothesized colonization routes of *J. genoveva* in Mexico; (b) Google Earth satellite view of a section of Estero del Soldado and the mangrove forest lining the inner shoreline. Arrows and dashed lines show the 0.5 km route used for weekly estimations of numbers of adult *J. genoveva*.

northward into Mexico from populations originating in Central or South America [3]. Here, we use several tests of COI sequence data from the two populations to obtain estimates of demographic histories in an attempt to provide further insight into the northward expansion hypothesis and the present-day distribution of *J. genoveva* in Mexico.

2. Materials and Methods

2.1. Sampling. Adults of *J. genoveva* from northwestern Mexico were collected at Estero del Soldado, near Guaymas, Sonora, Mexico (Figure 1(b)), and at nearby San Carlos [9]. One adult was reared on black mangrove from a first instar larva obtained at Estero del Soldado. Sample size was $N = 19$. Adults from the Costa Rica population ($N = 22$), listed as *J. evarete* [10], were obtained from larvae collected from the Area de Conservación Guanacaste (ACG), Guanacaste province in northwestern Costa Rica (Figure 1(a)) and reared on *Dyschoriste valeriana* or *Stachytarpheta jamaicensis*.

To estimate seasonal abundance of *J. genoveva* at Estero del Soldado, we conducted a weekly survey of adults from February 2011 to February 2012. Adults encountered on a 0.5 km dirt road adjacent to the mangrove forest (Figure 1(b)) were averaged over two-week intervals. The pattern of abundance (Figure 2) is in general agreement with Brown et al. [7] who found that *J. genoveva* (as *J. evarete*) in Baja California Sur, Mexico flies from September through February, but is most common from September through November. Although we cannot state with certainty the number of generations per year in *J. genoveva*, our data

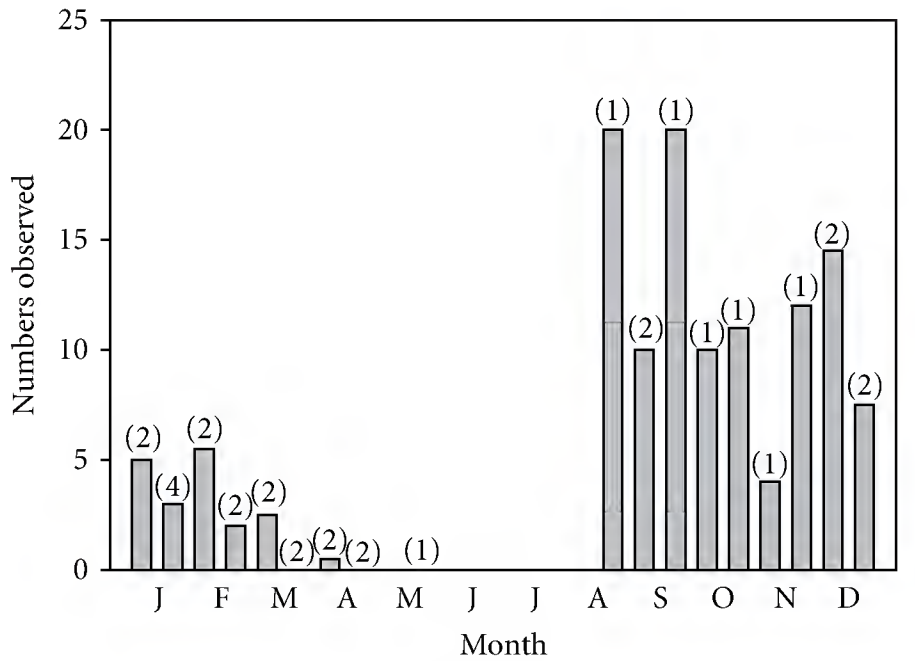


FIGURE 2: Numbers of adult *J. genoveva* observed flying along a 0.5 km route at Estero del Soldado averaged over two-week intervals (see Figure 1(b)). The number of observations made during each interval is shown in parentheses. Data were not taken during the first two weeks of May, or from June to mid-August.

are consistent with multiple broods. For the demographic tests, we have chosen a conservative value of two generations per year for the Guaymas population and have assumed the same value for the Costa Rica population (see Section 4).

2.2. Molecular Protocol. Details on standard procedures used for extraction of total genomic DNA from butterfly legs and amplification of the COI gene segment are found elsewhere

[3, 12]. The 658 bp segment corresponds to the COI barcode region [13]. GenBank accession numbers are JQ430692–JQ430710 (Guaymas, Mexico) and GU334034, GU334037, GU157280–GU157292, and GU157294–GU157300 (ACG, Costa Rica). Specimen voucher codes and geographic coordinates for corresponding GenBank accession numbers are found in Pfeiler et al. [3]. Detailed information on Costa Rica specimens can be obtained by entering voucher codes into the ACG database [10].

Twelve additional COI barcode sequences were available in GenBank for *Junonia* from four states in southern Mexico: Morelos (JQ430731, JQ430733), Campeche (GU659427, GU659432, GU659436), Yucatán (GU659429–GU659431), and Quintana Roo (HQ990188, GU659425, GU659426, GU659435). Although these sequences are currently assigned to *J. evarete*, our analyses showed that they belonged to the *J. genoveva* genetic lineage. These samples were not used in the demographic analyses owing to low sample sizes from each region, but their geographic distribution (Figure 1(a)) provides additional support for our colonization hypothesis.

2.3. Data Analysis. Calculations of genetic diversity indices and Tajima's [14] D were performed in DnaSP version 5.00.04 [15]. Fu's [16] Fs neutrality tests were conducted in ARLEQUIN version 3.5.1.3 [17] using 1000 simulations. In addition to assessing whether nucleotide polymorphisms deviate from expectation under neutral theory, Fu's Fs test is also useful for detecting signatures of population expansions, which lead to large negative values in the test statistic [16, 18]. The significance of Fs at the 0.05 level was indicated when P values were <0.02 [17].

Estimates of changes in effective female population size (N_{ef}) over time for *J. genoveva* were obtained in FLUCTUATE version 1.4 [19] and from Bayesian skyline analysis implemented in BEAST version 1.2 [20]. FLUCTUATE provides simultaneous maximum likelihood estimates of the mutation parameter (θ) and the exponential population growth parameter (g), where $\theta = 2N_{ef}\mu$, and μ is the neutral mutation rate per site per generation. To estimate μ, we assumed 2.3% pairwise sequence divergence per million years for the COI gene [21] and two generations per year, yielding a single lineage value of $\mu = 5.8\times10^{-9}$. Details on the program settings used in FLUCTUATE are given in Pfeiler et al. [22]. Bayesian skyline analysis utilizes Markov chain Monte Carlo (MCMC) sampling of sequence data to estimate a posterior distribution of effective population size through time [20]. Bayesian skyline analyses were run under the conditions of the HKY + Γ model (four gamma categories) using the mean mutation rate per site per generation (μ) described above. Ten million iterations of the MCMC chains were run, sampling every 1000 iterations. The Bayesian skyline plots were generated with TRACER version 1.2.1 [20]. To provide confirmation of population expansions detected in FLUCTUATE and Bayesian skyline analyses, demographic histories of both populations were also tested by analyzing the distribution of pairwise sequence differences, also known as the mismatch distribution [23, 24], using ARLEQUIN. The significance of the estimated parameters of the sudden expansion model of the mismatch distribution is obtained from the sum of square deviations (SSD) statistic and the raggedness statistic (rg), and their corresponding P values. The sudden expansion model is rejected when P is <0.05.

Table 1: Effective female population sizes (N_{ef}) and exponential growth rates (g) in *Junonia genoveva* from Costa Rica and Guaymas, Mexico determined with FLUCTUATE.

Locality	N	θ	N_{ef}	g ($1/\mu$ generations)
Costa Rica	22	0.1731 (±0.0511)	1.49×10^7	2603 (±231)
Guaymas	19	0.0065 (±0.0056)	5.60×10^5	3839 (±1895)

Values for maximum-likelihood estimates of θ and g (±1.96 standard deviations) are shown.
A neutral mutation rate per site per generation (μ) of 5.8×10^{-9} was assumed.

3. Results

Genetic diversity indices for the 658 bp COI segment reported previously [3] revealed that haplotype diversity (h) and nucleotide diversity (π) were higher in the Costa Rica population (h (±SD) = 0.926 ± 0.039; π = 0.00455 ± 0.00075; N = 22) than in the Guaymas, Mexico population ($h = 0.696 \pm 0.077$; $\pi = 0.00133 \pm 0.00023$; N = 19). Tajima's D values also were not significant in either populations. In the present study, a significant negative value for Fu's Fs was found for the Costa Rica population ($Fs = -7.61$; $P < 0.0001$). Although the value for the Guaymas population was not significant ($Fs = -1.52$; $P = 0.068$), it approached the $P < 0.02$ cutoff level for significance. The results suggest a historical population expansion for the Costa Rica population, which as we show below is consistent with results obtained from the other demographic tests. These tests also suggest a historical population expansion for the Guaymas population, but in all cases the evidence for this expansion is weaker than for the Costa Rica population.

The results from FLUCTUATE (Table 1) showed a positive exponential growth parameter (g) that was significantly different from zero in both populations of *J. genoveva*, indicating an increase in population size, although the standard deviation of g was much higher in the Guaymas population. Also, effective female population size (N_{ef}) was much larger in *J. genoveva* from Costa Rica than in the population from Guaymas (Table 1).

Bayesian skyline analyses (Figure 3) also indicated that both populations of *J. genoveva* have increased in size, with the present day N_{ef} again much larger in the Costa Rica population (Figure 3(a)). The timing of the expansion of the Guaymas population (Figure 3(b)) was estimated to be more recent (~86,000 years before present, BP) than the Costa Rica population (~400,000 BP).

Results from the mismatch distribution are shown in Table 2 and Figure 4. For the Costa Rica population (Figure 4(a)), the plot of the observed distribution of pairwise differences among COI haplotypes was unimodal and agreed well with the expected distribution for a population that has undergone an expansion. A unimodal curve also was seen in the Guaymas population, but the fit with the expected distribution was not as good (Figure 4(b)). For both

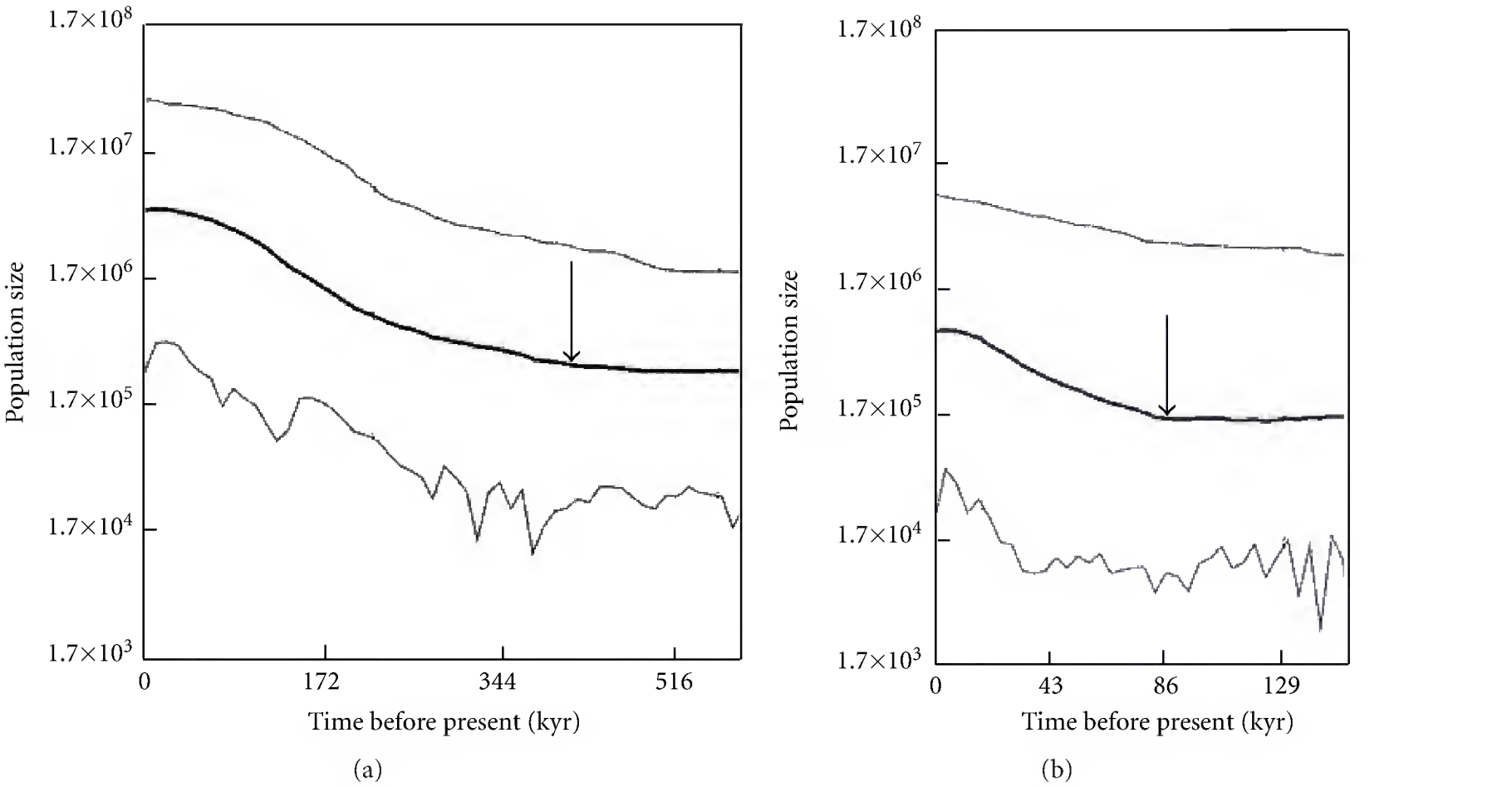


FIGURE 3: Bayesian skyline plots showing changes in effective female population size (N_{ef}) over time for *Junonia genoveva* from Costa Rica (a) and Guaymas, Mexico (b). Population size is given on a logarithmic scale. The thick solid lines represent the median estimates of population size; the thin solid lines show the 95% HPD (highest posterior density) intervals. Note the different time scales in the two plots. Arrows show estimated dates for the beginning of both population expansions.

populations, the test statistics SSD and rg were small and not statistically significant (Table 2), indicating that the sudden expansion model could not be rejected for either population. The P values for SSD and rg for the Guaymas population, however, were near the 0.05 cutoff level for significance (Table 2), suggesting that evidence for population size increase is weaker in this population, consistent with the results from FLUCTUATE.

Based on the results from the mismatch distribution (Table 2), we estimated the timing of the population expansion in both the Costa Rica and Guaymas populations of *J. genoveva* using the equation $\tau = 2ut$, where τ is a moment estimator of mutational time, t is the number of generations since the expansion, and u is the mutation rate for the entire gene segment of 658 bp [23]. Assuming 2.3% pairwise divergence per million years [21], the mean mutation rate per site per generation in the 658 bp segment for a single lineage is equal to $(658) \times (1.15 \times 10^{-8})$ or 7.57×10^{-6}. The estimated time to the population expansion in the Costa Rica population (with 95% confidence intervals) was 132,450 (52,318–375,500) generations ago, and was 70,199 (13,245–145,030) generations ago in the Guaymas population. Although the confidence intervals are large, these calculations reveal the same trend as seen in Bayesian skyline analysis in which the timing of population expansion in the Costa Rica population predates that of the Guaymas population.

4. Discussion

Our results show that female effective population size (N_{ef}) and genetic diversity indices are larger in the Costa Rica population of *J. genoveva* compared to the northwestern Mexico population at Guaymas, consistent with the hypothesis that the Guaymas population was founded by migrating individuals from Central America as depicted in Figure 1(a). In addition, the presence of *J. genoveva* throughout the Yucatán Peninsula and the state of Morelos revealed by barcode analysis of available GenBank sequences (listed as *J. evarete*) suggests that colonization from Central America source populations was widespread in Mexico. Additional sampling will be required to determine if the *J. genoveva* lineage also colonized northeastern Mexico and southern USA, but this scenario seems probable based on the data presented here.

Population expansions in the Guaymas and Costa Rica populations of *J. genoveva* both dated to the Pleistocene, but differences in the timing of the expansions (Figure 3) are also consistent with the conclusion that the Guaymas population is the more recent. These differences, however, are only rough estimates that depend on the assumption of a standard 2.3% molecular clock and two generations per year in both populations. Different numbers of generations per year would result in changes in the estimated timing of the expansions, but it is unlikely that our conclusions would be affected. For example, if we assume a value of three generations per year in the Costa Rica population, the estimated time of the expansion would increase by about 50% to ~600,000 years BP, still within the Pleistocene and much earlier than the population expansion at Guaymas (~86,000 BP). In an unlikely scenario of one generation per year in the Costa Rica population and three generations per year at Guaymas, the Costa Rica population expansion

Table 2: Results of the mismatch distribution of COI sequences in *Junonia genoveva* from Costa Rica ($N = 22$) and Guaymas, Mexico ($N = 19$).

Population	τ (95% CI)	θ_0	θ_1	SSD	rg
Costa Rica	2.00 (0.79, 5.67)	1.11	58.13	0.0019 ($P = 0.81$)	0.024 ($P = 0.81$)
Guaymas	1.06 (0.20, 2.19)	0.00	>1000	0.0299 ($P = 0.08$)	0.197 ($P = 0.06$)

τ: moment estimator of mutational time; θ_0 and θ_1: mutation parameters where $\theta_0 = 2uN_0$ and $\theta_1 = 2uN_1$ (N_0 and N_1 are the population sizes before and after the expansion, resp.) with 95% confidence intervals (CI); SSD: sum of square deviations; rg: raggedness statistic [23, 24].

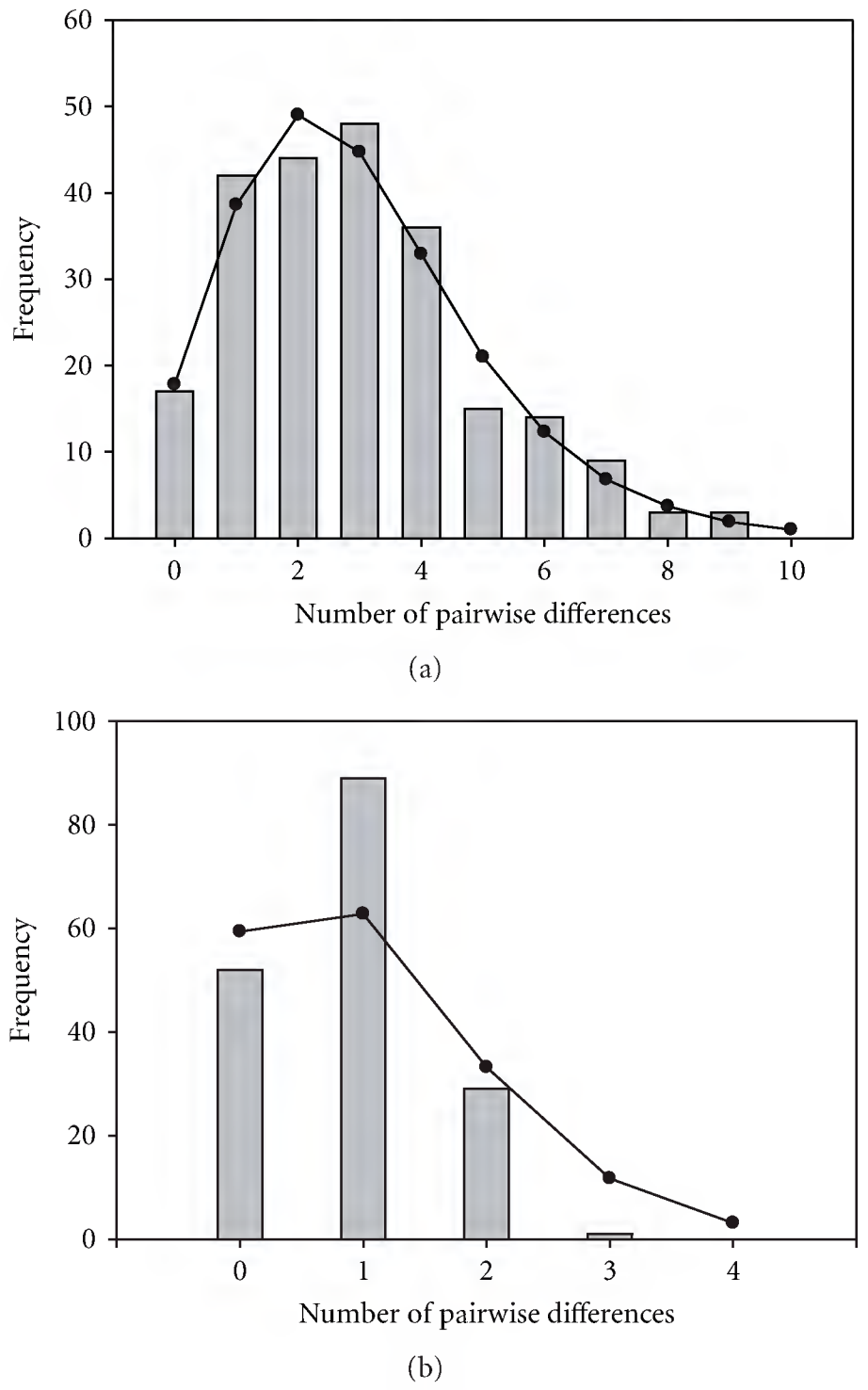


Figure 4: Distribution of pairwise differences among COI haplotypes (mismatch distribution) in populations of *Junonia genoveva* from Costa Rica (a) and Guaymas, Mexico (b). Solid lines represent the expected distributions under the sudden expansion model.

would still predate the Guaymas expansion (~200,000 and ~130,000 years BP, resp.).

The apparent absence of *J. evarete* in Mexico and Central America as suggested by barcode analysis is noteworthy, as this species is reported to be a common inhabitant of the region [6–8]. More extensive sampling may ultimately reveal *J. evarete*, but all COI barcodes obtained to date ($N = 88$), from samples covering a broad geographic area from northwestern Mexico to Panama, and including both coastal and inland populations utilizing different larval host plants, clearly belong to the *J. genoveva* lineage ([3], present study).

Specimens of *Junonia* from Baja California Sur listed as *J. evarete* [7, 8] show an ecological association with the mangrove habitat, in addition to showing similar patterns in wing color and seasonal abundance compared with the Guaymas population, leading to the suggestion that they should be reassigned to *J. genoveva* [3, 9]. Given this evidence, we show colonization of Baja California Sur by *J. genoveva* on Figure 1(a), although no COI barcodes are available for confirmation. Our demographic evidence suggesting a late Pleistocene origin for the mainland population at Guaymas would also imply that putative colonization of *J. genoveva* in Baja California Sur occurred by over-water dispersal from the mainland, as the separation of the Cape Region of the Baja California peninsula from the mainland is thought to have occurred during the Pliocene, about 3-4 Ma [25]. In support of this hypothesis, *J. villida* (Fabricius), a species closely related to the New World *Junonia* [1], is known to be a strong over-water disperser, showing a broad geographic distribution among Pacific islands [26, 27].

Acknowledgments

The authors thank T. Hernández Mendoza and M. Polihronakis-Richmond for help with this project. They are especially grateful to D. H. Janzen, W. Hallwachs and N. Wahlberg for kindly sharing their unpublished data. They also thank R. A. Bailowitz, C. Brévignon, J. Calhoun, and K. Hansen for helpful discussions and providing insights into the taxonomy of *Junonia*. This research was supported by NSF Grant DEB-0346773 to T. A. Markow.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 396594, 10 pages
doi:10.1155/2012/396594

Research Article

Variation in the Stabilimenta of *Cyclosa fililineata* Hingston, 1932, and *Cyclosa morretes* Levi, 1999 (Araneae: Araneidae), in Southeastern Brazil

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Received 5 April 2012; Accepted 10 May 2012

Academic Editor: Martin H. Villet

We investigated the characteristics of the stabilimenta constructed by two species of *Cyclosa*, describing the variations within and among five populations. Both species constructed stabilimenta composed entirely of silk (linear and spiral types) or of silk and debris (linear, detritus clusters and complex types). The vertical linear detritus type was the most frequent structure for adult females of both species, whereas stabilimenta consisting of detritus clusters were more frequent for juveniles of *C. morretes*. The latter structures appeared to be an intermediate state towards the linear continuous type usually found in adults. The other types were rarely found, and silk stabilimenta were to be constructed only when detritus was not available. The substitution of silk by detritus indicated that both materials function as camouflage in *C. morretes* and *C. fililineata* webs. The positions occupied by the spiders within the detritus column (and in some cases the orientation of the stabilimenta) varied markedly within populations, and the unpredictability of their location could be important in reducing the risks of predation. The hypothesis that stabilimenta constitute defensive devices was indirectly corroborated by the observation that spider's body width and length were, respectively, strongly correlated with the width and length of the stabilimenta.

1. Introduction

Stabilimenta are structures of silk or detritus included by some orbweaver spider species on the hub of their orbs or on the frame and anchor lines of the webs. These structures have appeared independently at least nine times during the evolution of orb-web spiders, always in species with webs that are exposed during the day [1, 2]. Herbestein et al. [2] found reports of web decorations in 22 genera of the families Araneidae, Tetragnathidae, and Uloboridae, but new records are continuously being added to this list (e.g., *Allocyclosa* [3, 4], *Molinaranea* [5], *Metepeira* [6], *Verrucosa*—M. O. Gonzaga, pers. obs.). Some stabilimenta, such as those of certain species of *Micrathena* and *Gasteracantha* (Araneidae), are generally composed of small silk flocks and are, therefore, considered by some authors to be nonfunctional structures [7]. Eberhard [8] argued that the placement of these silk flocks in the resting webs of *Gasteracantha cancriformis* and *Philoponella vicina* (Uloboridae) represent a strong evidence against prey attractant hypothesis for these structures but is compatible with the hypotheses of camouflage and web advertisement. Other stabilimenta, such as those of *Argiope* (Araneidae), are very conspicuous and have different shapes and sizes, depending on the age [9], size [10], and nutritional condition [11, 12] of the spiders.

Several species of *Argiope* show considerable within-species ontogenetic variation in the forms and frequency of decorations. Juveniles of *Argiope savignyi*, for example, usually construct discoid decorations, whereas adult females construct cruciate stabilimenta more often [7]. Schoener and Spiller [10] found that *A. argentata* of intermediate size build cruciate stabilimenta at a higher frequency than small and large individuals. These authors argued that the cruciate stabilimenta may serve to increase the apparent size of the spiders. Since lizards are major predators in their study areas (islands in Bahamas) and are gape-limited, spiders with a large apparent size may be less susceptible to attacks. Thus, large spiders would not need to appear still larger,

and small spiders would not build cruciate structures in order to avoid appearing like medium-sized spiders, which are a suitable prey for lizards. Li and Lee [13] showed that stabilimentum building and other web traits of *Argiope versicolor* change in response to the risk of predation. In that case, the presence of predator chemical cues induces the reduction stabilimentum area and frequency of construction by juveniles. The proportion of individuals that decorate their webs can also vary among populations. Hauber [14], for example, showed that only about 25% of *A. appensa* individuals from Guam built stabilimenta, whereas Kerr [15], who studied other populations of this same species on neighbouring Pacific islands, reported frequencies of web decorations that varied from 4% to 76%.

In the genus *Cyclosa*, most of the known species add web threads, debris (which is composed mainly of prey remains), and egg sacs to the central region of the webs to form linear continuous [16], linear discontinuous [17], spiral [18], or more complex structures (M. O. Gonzaga, pers. obs.). According to Nentwig and Heimer [7], each species has a specific stabilimentum pattern, but there have been few reports of intraspecific variation [2, 3, 16]. This variation may be very important when assessing the function of these structures. Craig [19, 20], for example, showed that the stingless bee *Trigona fulviventris* can learn to avoid the webs of *A. argentata* decorated with the same pattern of stabilimentum over successive days. When the orientation of these structures was varied, however, bees were more likely to be caught in the web threads. This finding indicates that an unpredictable decorating behaviour may reduce the probability that potential prey and/or predators and parasitoids will associate a specific pattern with the presence of a web.

The aims of this study were to determine whether there are species-specific patterns of stabilimenta in two *Cyclosa* species from southeastern Brazil and to describe the intraspecific variation of this structure within and among populations. We also tested whether stabilimentum measurements (width and length) follow the body size of their builders. The similarity between stabilimentum width and spider body width may be important to disrupt the contour and shape of spiders by blending individuals with the shape of the column of detritus, therefore reducing the probability of location by visually oriented predators. Likewise, increasing the length of the linear detritus structure during maturation, spiders may reduce the success of predators in locating their bodies within the stabilimentum. Finally, we investigated the process of reconstruction of stabilimenta after the removal of the original structure in order to assess whether some previously identified types of decorations were simply intermediate stages during the construction of other types.

2. Material and Methods

The samples were collected in five areas in southeastern Brazil, by visually searching along forest borders and within forests. The reserves ARIE Floresta da Cicuta (22°32′39″ S 44°05′22″ W), Parque Nacional Itatiaia (22°26′44″ S 44°36′43″ W), and Parque Estadual Intervales (24°16′38″ S 48°25′07″ W) are composed of old secondary growth and primary evergreen cloud forests (Mata Atlântica). In the Parque Estadual da Ilha do Cardoso (25°04′19″ S 47°55′30″ W), *Cyclosa fililineata* individuals were collected from cloud forests and the coastal sand dune vegetation (Restinga). The predominant vegetation types in Fazenda Rio Claro (22°46′22″ S 48°52′58″ W) are *Eucalyptus* plantations and secondary growth subtropical humid forests. Each area was sampled three times, with an interval of three months between consecutive surveys. This time interval was used to ensure that individuals in different stages of maturation were sampled.

The width (close to spider's position) and total length of the column of detritus, as well as the length of the upper and lower segments of the stabilimenta were measured for all of the webs that were located. Spiders and their stabilimenta were collected and latter measured in the laboratory using a dissecting microscope fitted with an ocular micrometer. To ensure that the dimensions of the stabilimenta remained unaltered during transportation, these structures were fixed to cardboard with a thin layer of glue.

The data obtained were used to compare the characteristics of stabilimenta between species and among populations and to calculate an index of symmetry of the detritus column using the equation: $IS = (((E/2) - A)/E) * 2$ (where E is total length of the column and A is distance between the spider and the upper extremity of the column). This index varied between -1 and 1. An index of 0 meant that the spider was located exactly in the middle of the column. In addition to the symmetry of the stabilimenta, we also recorded the inclination of these structures using a circular grid positioned behind the spider.

The Mann-Whitney U-test was used to compare the adjustment of abdominal width of the spiders with the width of the detritus column between *Cyclosa* species. The adjustment was calculated by subtracting the largest width of the abdomen from the width of the stabilimentum at the position immediately above the hub of the web. The relationship between abdominal width and the width of the stabilimentum and between spider body length and the length of the decorations were investigated by regression analysis using the pooled data of all populations for each species.

We also removed the stabilimenta of 56 individuals of *C. fililineata* and 48 individuals of *C. morretes* to study the process of rebuilding. For this, we completely destroyed the original webs and retained only the bridge thread, from which the spider started the construction of a new web. The web and stabilimenta parameters mentioned above were measured for the original web and at 24 h intervals after removal of the stabilimentum. These spiders were followed for a period of 120 h.

3. Results

Cyclosa fililineata and *C. morretes* built basically the same five types of stabilimenta: linear structures containing silk and

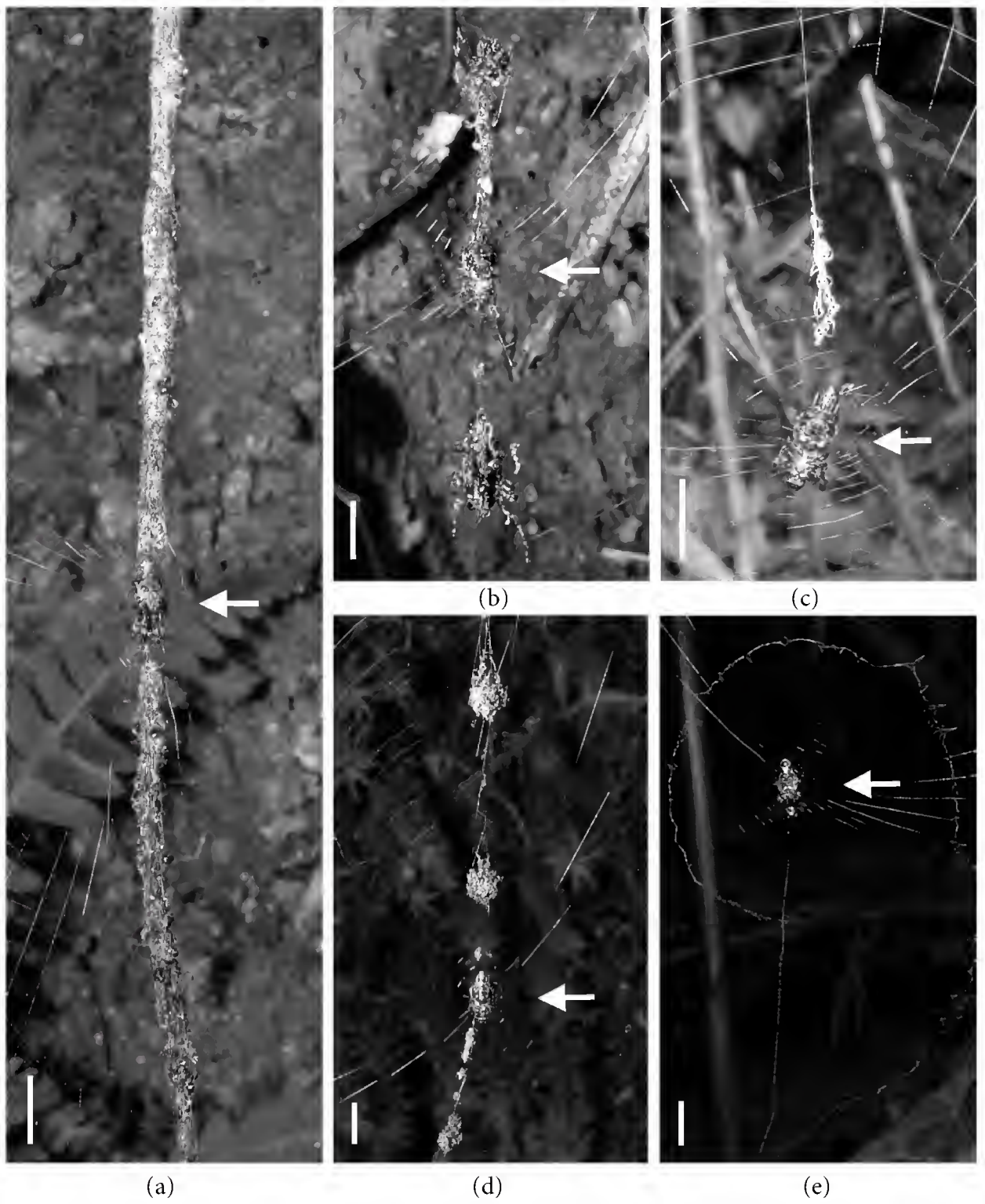

FIGURE 1: Decoration patterns in *C. morretes* and *C. fililineata* webs. (a) Linear detritus structure, (b) complex type with a linear segment and a blob resembling a large spider, (c) linear silk structure, (d) blobs of detritus forming a linear discontinuous structure, and (e) silk spiral. The arrows indicate the position occupied by the spider. Scales: 5 mm.

debris, incomplete columns characterized by one to many blobs of silk and debris, complex types (often resembling a large spider), spiral shapes composed only of silk, and linear silk structures (Figure 1). Adult females of both species constructed linear detritus columns more frequently than any other type of stabilimentum. However, linear discontinuous stabilimenta were very often found in webs of immature individuals of *C. morretes*. Complex detritus structures and both types of silk stabilimenta occurred only rarely in all of the populations studied (Figure 2).

The adjustment between the abdomen of the spiders and their stabilimenta was different for the two species (Mann-Whitney, $U = 6329.0, n_{C.\,fililineata} = 287, n_{C.\,morretes} = 80, P < 0.001$), with *C. fililineata* showing a better adjustment. The mean difference between the spiders and their stabilimenta in this species was 0.21 ± 0.15 mm, while in *C. morretes* the mean difference was 0.47 ± 0.37 mm. The dimensions of the stabilimenta varied with spider size. The width of the column close to the spider's position was strongly correlated with the abdominal width in both species ($r^2 = 0.49, F = 273.6, P < 0.001, n = 287$ for *C. fililineata* and $r^2 = 0.60, F = 118.1, P < 0.001, n = 80$ for *C. morretes*). Similarly, the length of the column was correlated with spider body length ($r^2 = 0.48, F = 368.5, P < 0.001$ for *C. fililineata*, and $r^2 = 0.42, F = 68.9, P < 0.001$ for *C. morretes*) (Figure 3).

The symmetry of the stabilimenta varied in all of the populations studied, especially in webs belonging to *C. fililineata*. Nevertheless, the extremities of the columns were always occupied less frequently than the central positions in this species (Figure 4(a)). *Cyclosa morretes* generally occupied the lower extremity of the column in Parque Nacional Itatiaia and in Parque Estadual Intervales. In Floresta da Cicuta,

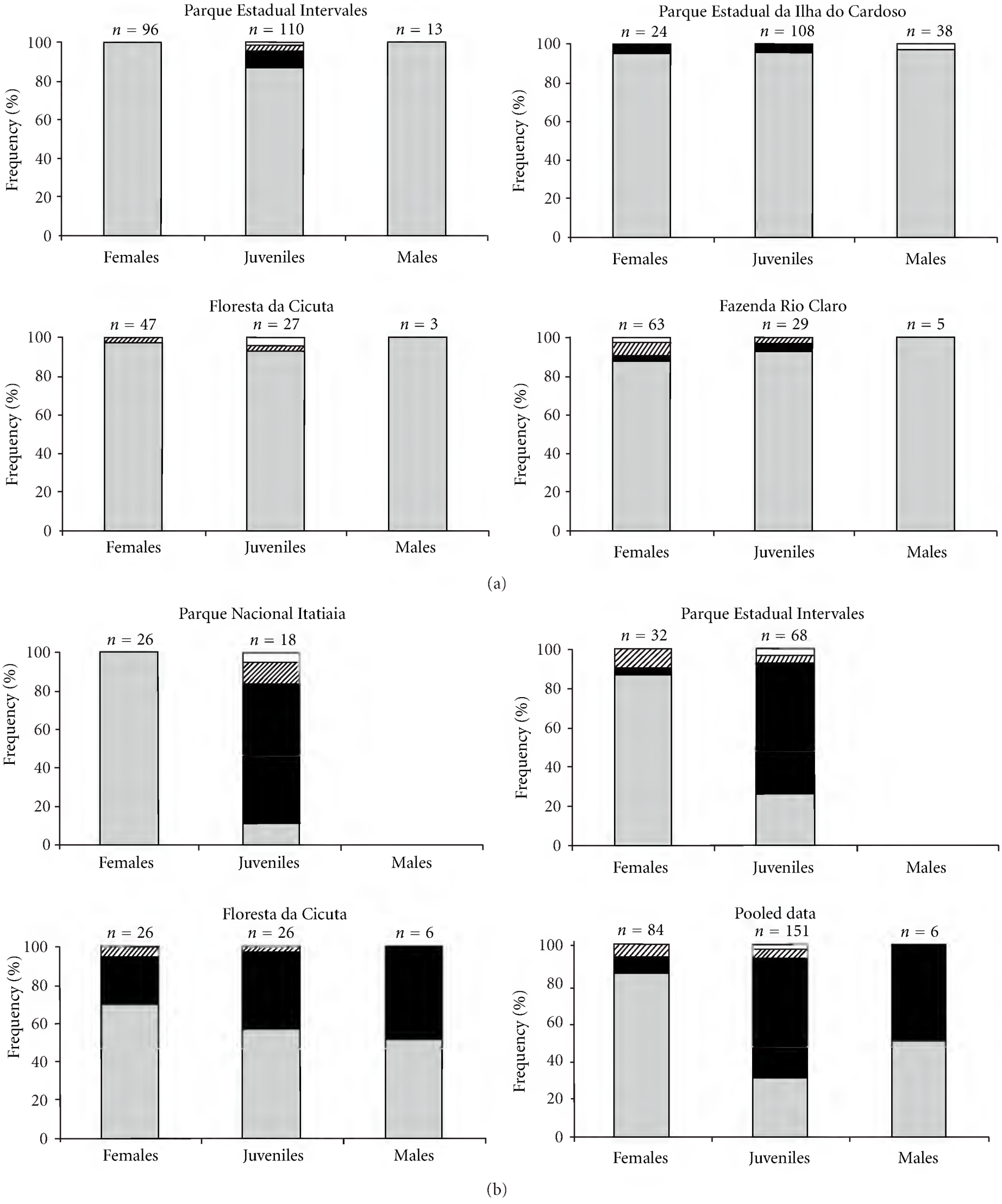

FIGURE 2: Frequencies of web decorations constructed by (a) *C. fililineata* and (b) *Cyclosa morretes* in the study areas. Grey bars: linear structures with detritus; black bars: linear discontinuous structures with detritus (blobs); bars with diagonal lines: complex types with detritus; white bars: linear silk + spiral silk stabilimenta.

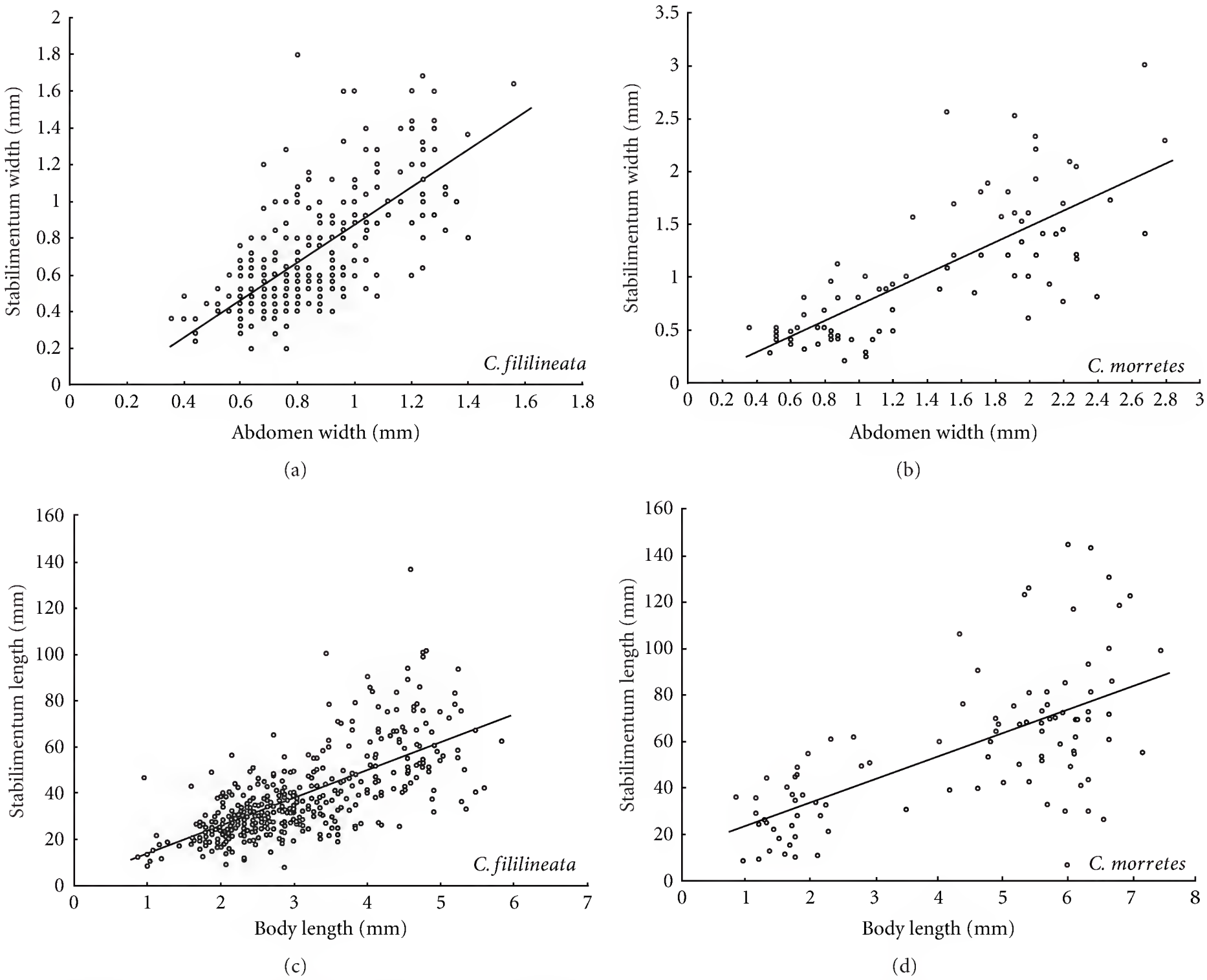

Figure 3: Relationship between abdominal width and the width of the linear detritus stabilimenta constructed by (a) *C. fililineata* and (b) *C. morretes;* and between body length and the length of the linear detritus stabilimenta constructed by (c) *C. fililineata* and (d) *C. morretes*. The points represent the pooled results for adults and juveniles from all populations.

however, we found several individuals in the lower extremity, but also a large proportion in central positions (Figure 4(b)). In all populations, most stabilimenta were constructed in a vertical position, but this orientation was not obligatory for these species (Figure 5).

The pattern of the original stabilimentum, in most cases a linear structure with detritus, was reconstructed within 72 h in most of the webs of *C. fililineata* (Figure 6). Several webs constructed the day after the removal of the original stabilimentum contained otherwise rare linear and spiral silk structures. The presence of silk stabilimenta on the first day after web destruction was also seen in *C. morretes*. However, in this species the frequency of linear detritus structures, even after 120 h, was still relatively low. Linear silk stabilimenta were gradually substituted by detritus as soon as the spiders had access to prey items or had collected debris intercepted by the web, but many individuals of *C. morretes* usually placed detritus far from the position occupied by the spider, creating discontinuous structures (Figure 7).

4. Discussion

All of the types of stabilimenta observed here in webs of *C. fililineata* and *C. morretes*, except for the complex shape with detritus, have been described for other species of this genus [3, 17, 18, 21]. Our comparisons among populations showed that the proportions of these types were similar for each species at different localities. Linear detritus structures were the commonest type constructed by *C. fililineata* and also by females of *C. morretes*. Unlike the discontinuous columns (blobs of detritus), this shape completely disrupted the visual sign of a spider. This could be an indication that the function of these stabilimenta is related to protection, possibly by reducing the probability of the spider being located by visually oriented predators. This disruptive function was supported by our results that showed a relationship between the stabilimentum width and the width of the spider's abdomen. The width of the column was generally similar to the size of the spiders, which helped to conceal their

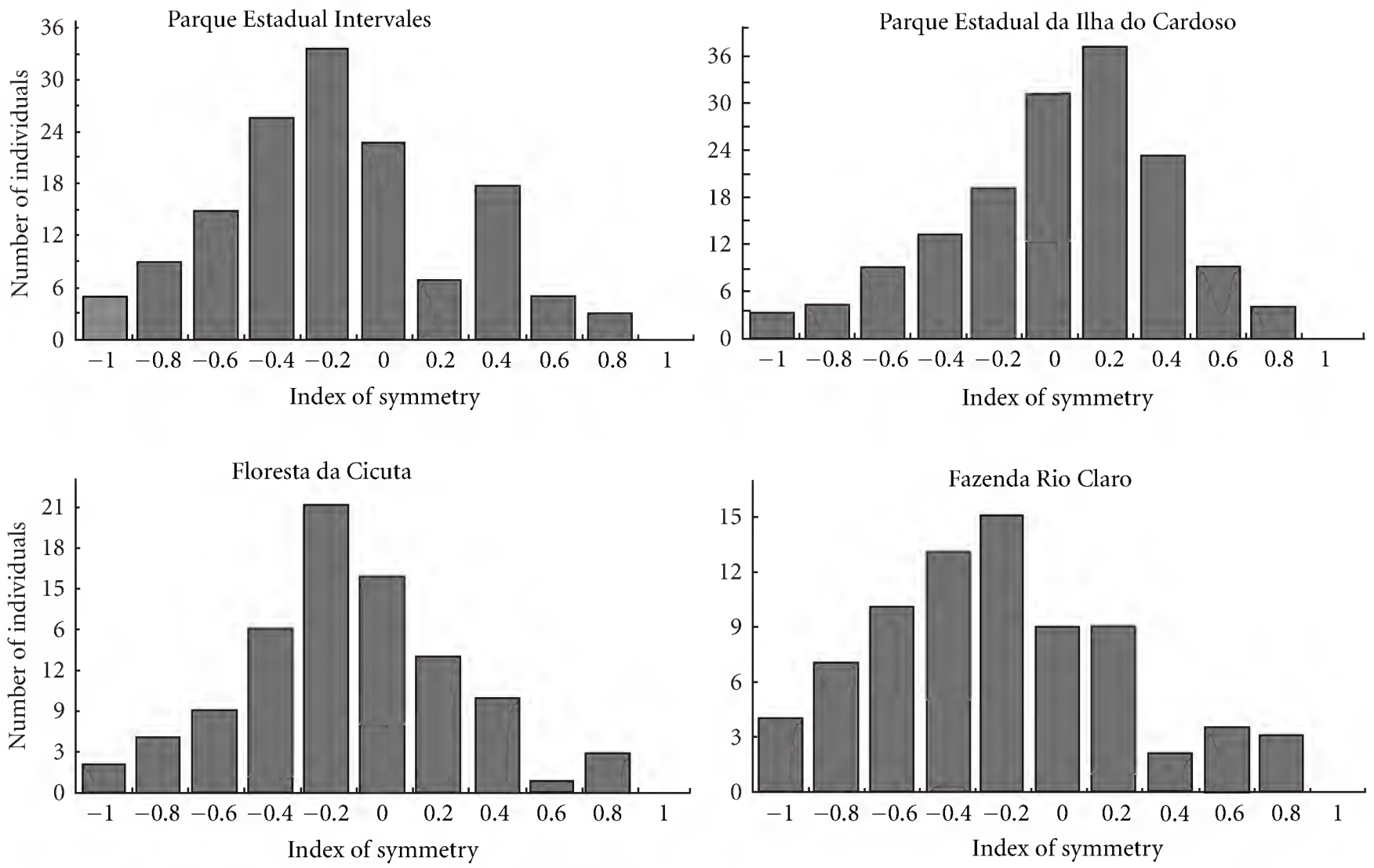

FIGURE 4: Positions occupied by adult (a) *C. fililineata* and (b) *C. morretes* in the detritus column in each population. −1: lower extremity, 0: center, +1: upper extremity.

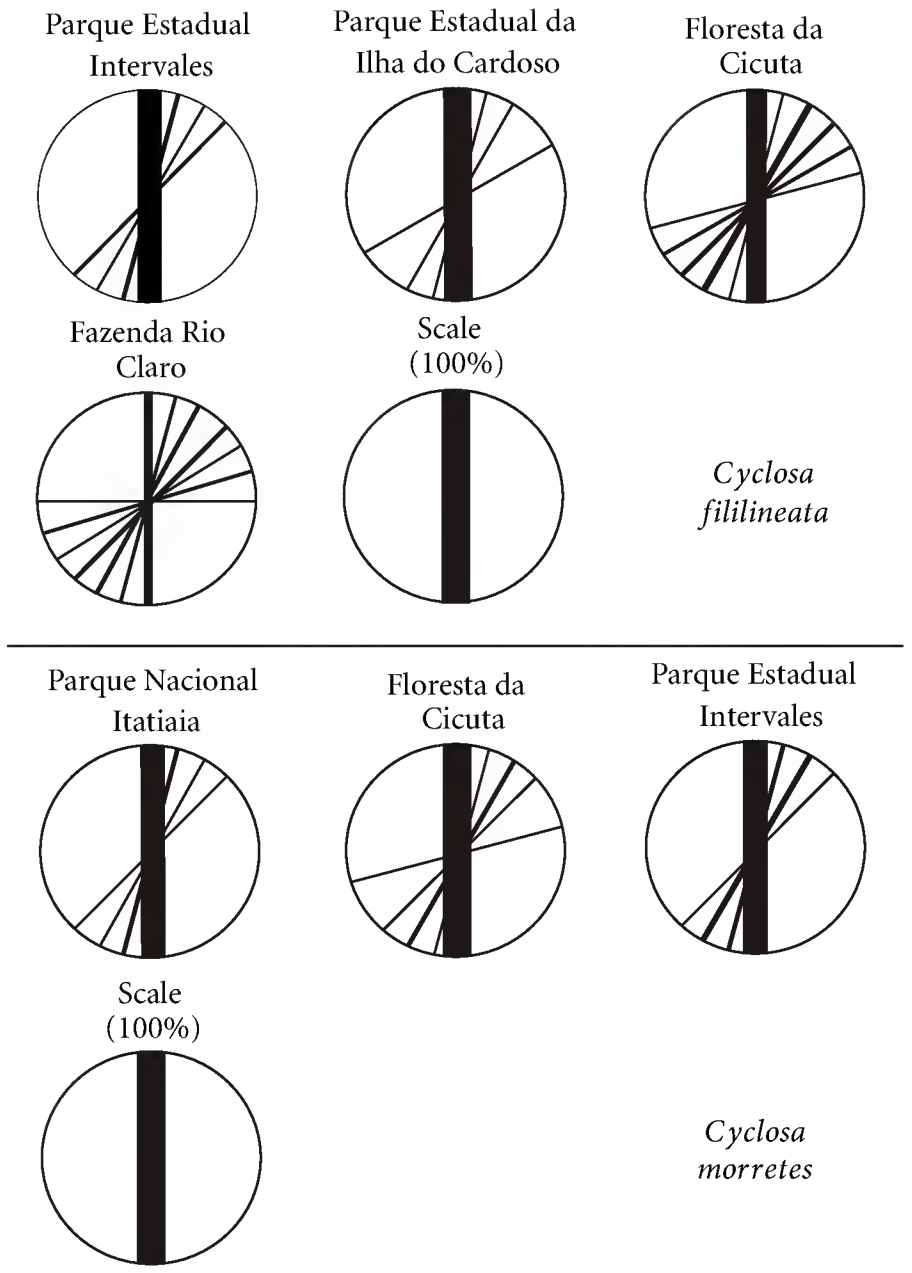

FIGURE 5: Frequencies of the inclinations of the stabilimenta constructed by *C. fililineata* and *C. morretes*. The inclinations were measured at 15° intervals and the thickness of each bar indicates the percentage of each category found in the field. Pooled results for juveniles and adults.

positions. Gonzaga and Vasconcellos-Neto [22] showed that artificial spider models (with about the same size of *C. morretes* females), attached to threads of nylon, were more attacked in the field than similar models in threads containing segments of modelling clay simulating stabilimenta. These results confirm that some predators were searching for prey items with the shape of a *Cyclosa* body and that columns with the same width of the spider body could reduce the risks of predation. Large spiders also build long columns, which is probably a consequence of the time they had to accumulate detritus and the availability of detritus after the consumption of relatively larger prey items. Long columns of detritus may be more efficient in reducing the probability of precise attacks by predators at the positions occupied by the spiders.

The placement of blobs of detritus, however, may also be a good strategy to avoid predators, especially when spiders have to deal with a very limited amount of detritus. The blobs were frequently about the same size as the spider and predators may be confused by this similarity during attacks. If the wrong target is attacked (a blob located far from the spider's body), the spider may have time enough to escape by running or jumping from the web. The complex type of stabilimentum appears to be a derivation of a blob but is larger and frequently contains projections resembling legs. We still do not have information on detritus acquisition rate and the suitability of particular types of debris to confirm the hypothesis that these types are associated to the availability of material to stabilimenta construction. Future work on these questions and comparing the survivorship of spiders with different stabilimentum types are important to determine the effectiveness of these structures against predators.

The variation in stabilimentum symmetry within several of the studied populations may be an indicative of a level of unpredictability that may be important in avoiding predators. There is no field evidence that spider predators use the stabilimenta to locate their prey [23]. Nevertheless, if this eventually occurs, the predators would have to locate the spider in the middle of the detritus column. By always attacking a specific position, predators will fail to capture prey most of the time. However, it is not clear why *C. morretes* occurs more frequently in the lower extremity of the detritus column. This position may allow a faster response after the detection of vibrational stimuli in the web, but we have no data to support or refute this hypothesis.

Another component of variability detected in this study was the orientation of the stabilimenta. Although most structures were constructed in a vertical position, there were stabilimenta that deviated from this direction in all of the studied populations. According to McClintock and Dodson [18], this variation in *C. insulana* may be related to the predominant orientation of elements in the background, thereby reducing the visibility of the decorations. In contrast, Rovner [17] showed that the orientation of the stabilimenta constructed by *C. turbinata* was determined exclusively by geotaxis. These divergent studies indicate that the causes and significance of variation in the direction of stabilimenta remain to be appropriately tested.

Herberstein et al. [2] argued that extrapolations from one phylogenetic group to another are unlikely to be relevant in resolving the debate about the functions of web decorations and that structures containing debris probably should not be considered as "decorations" or "stabilimenta," but as a separate behavioural phenomena. However, our results showed that the linear structures containing detritus constructed by at least two *Cyclosa* species were initially composed of only silk and were very similar to the linear stabilimenta of many uloborids. Eberhard [4] described the same pattern of substitution of one type of structure for another in *Allocyclosa bifurca* and in *Cyclosa monteverde*. In addition, he showed that these species do not construct silk stabilimenta when egg sacs are available. These findings suggest that the possible function of silk decorations can also be fulfilled by a structure containing egg sacs or detritus and that these different devices are variations of the same behavioural unit.

The observation that detritus is placed over silk stabilimenta (but never the opposite) argues against the hypothesis that the latter devices are used to attract prey. Detritus probably would interfere with any reflective property of the silk stabilimenta, thereby reducing their effectiveness in attracting insects searching for UV signals. Alternatively,

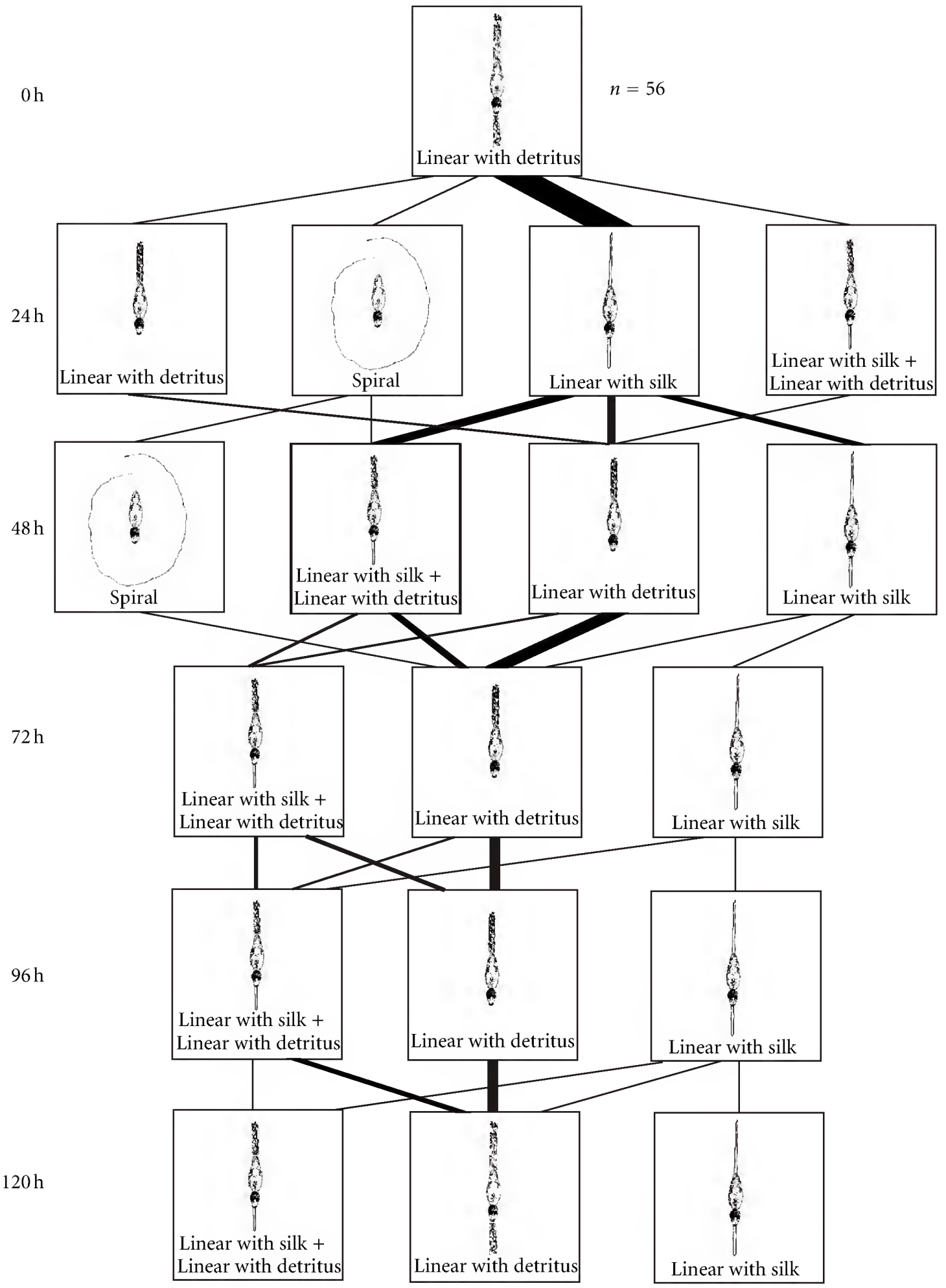

FIGURE 6: Reconstruction of stabilimenta by *C. fililineata*. The thickness of the lines indicates the number of webs that passed to the next stage.

linear and spiral silk stabilimenta may also disrupt the spider's shape but are soon replaced because they are probably less effective. At least to human eyes, spiders are easier located within silk stabilimenta.

Neet [24] argued that the spiral stabilimenta constructed by *C. insulana* may confer mechanical stability to orb-webs under strong wind. However, this conclusion was based solely on the observation that spiral stabilimenta were constructed at a higher frequency after stormy nights with strong winds. This observation could simply be a consequence of the higher incidence of web destruction under these weather conditions. Upon losing their original linear stabilimenta, spiders cannot build immediately another similar device because they do not have the detritus to incorporate into the webs. The hypothesis of mechanical stability must be tested by submitting webs with spiral and linear silk stabilimenta to controlled wind intensities and by measuring their resistance.

Acknowledgments

The authors are grateful to Natália O. Leiner, Paulo S. Oliveira, Glauco Machado, Andrea L. T. Souza, André L.

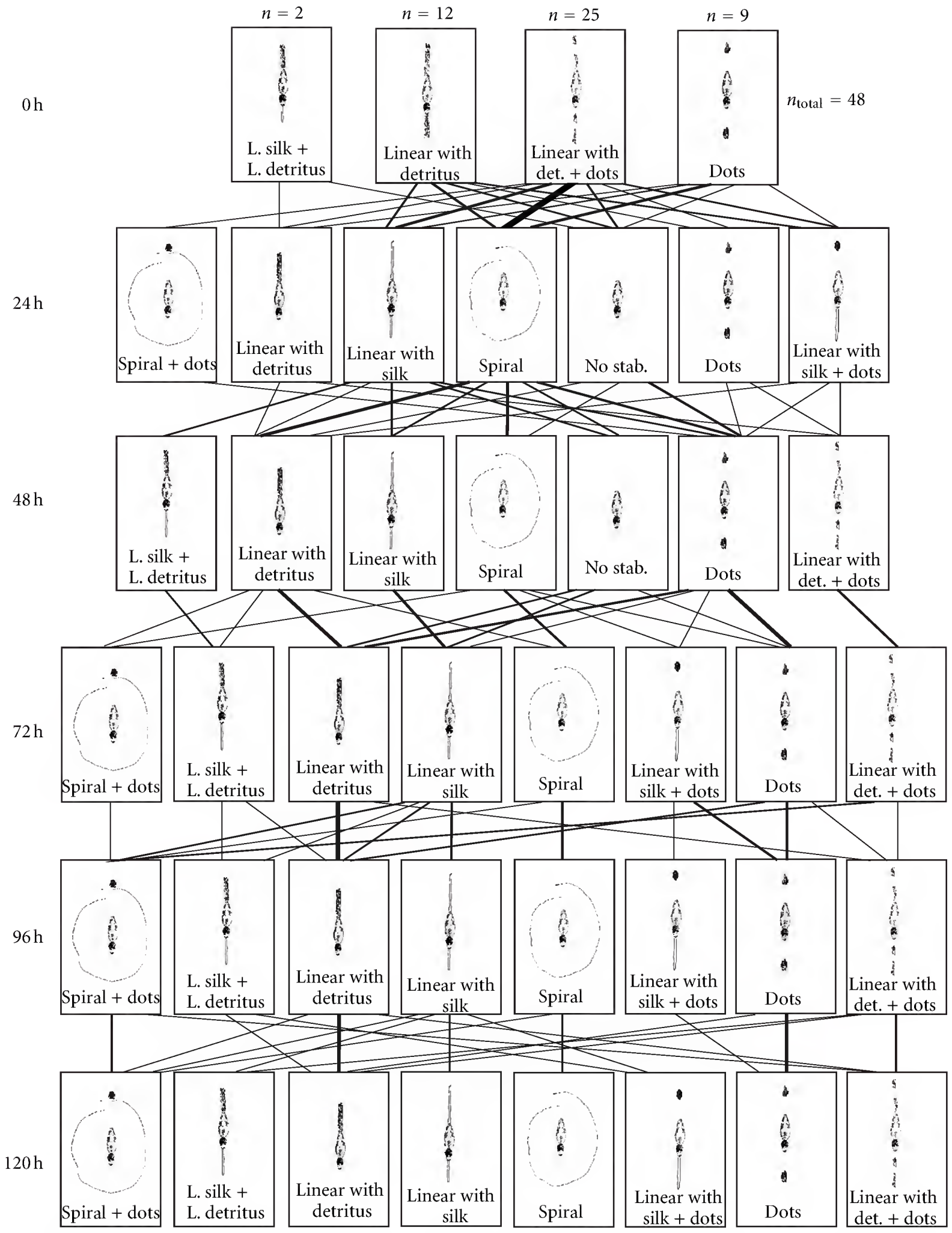

FIGURE 7: Reconstruction of stabilimenta by *C. morretes*. The thickness of the lines indicates the number of webs that passed to the next stage.

Freitas, and Hilton F. Japyassú for helpful suggestions on the paper, and to William G. Eberhard for fruitful discussions on stabilimenta. They also thank Fundação CSN, Fundação Florestal do Estado de São Paulo, Dutarex, and IBAMA for allowing their studies in the reserves. This study was financially supported by FAPESP (Proc. 99/06089-4 to M. O. Gonzaga), CNPq (Proc. 300539/94-0 to J. Vasconcellos-Neto), and Instituto Nacional de Ciência e Tecnologia dos Hymenoptera Parasitóides da Região Sudeste Brasileira (HYMPAR/Sudeste-CNPq/CAPES/Fapesp).

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 868474, 7 pages
doi:10.1155/2012/868474

Research Article

A Male Aggregation Pheromone in the Bronze Bug, *Thaumastocoris peregrinus* (Thaumastocoridae)

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Received 11 January 2012; Accepted 27 April 2012

Academic Editor: Jeffrey R. Aldrich

Forest plantations in Uruguay have doubled in the past decade, with *Eucalyptus* spp. leading this growth. The bronze bug, *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae), originally restricted to Australia, is an important emerging pest of *Eucalyptus* plantations in the Southern hemisphere. *T. peregrinus* feeds on mature *Eucalyptus* leaves, causing them to turn brown and often fall from the tree. Although population dynamics and behavioural patterns are not clearly understood, circumstantial observations suggest that males and nymphs aggregate. We used gas chromatography coupled to mass spectrometry to analyze volatile organic compounds emitted by virgin males and females, and characterized a male-specific compound, 3-methylbut-2-enyl butanoate, based on mass spectral data and chromatographic comparison with a synthetic standard. We also performed Y-olfactometer bioassays to test the attraction of virgin males and females toward live virgin males, male volatile extracts, and synthetic 3-methylbut-2-enyl butanoate. Males were attracted toward conspecific males, while virgin females showed no preference, suggesting that male volatiles are not involved in sexual communication. Further olfactometer tests showed that males were attracted to male volatile extracts and to synthetic 3-methylbut-2-enyl butanoate. The ecological significance of this compound and its potential use for the management of *T. peregrinus* in *Eucalyptus* forests will be further investigated.

1. Introduction

The area of commercial forests in Uruguay reaches about 1 million hectares, of which more than 70% are covered by *Eucalyptus* plantations. This area has nearly doubled in the past decade, as part of broader trend in South America. In Uruguay, *E. globulus* and *E. grandis* are the most important species, representing 54% and 32% of the *Eucalyptus* planted area, respectively, while red gum trees such as *E. tereticornis* occupy about 7% [1]. Depending on whether the plantations are intended for pulp or timber production, the growth cycles take between 8 to 12 years, being, therefore, a clear example of monocultural practice of an exotic crop, which should favor the spread of alien invasive pests and diseases, such as the bronze bug, *Thaumastocoris pereginus* (Hemiptera : Thaumastocoridae).

T. peregrinus is a major emerging pest of eucalypt production in the Southern hemisphere. It is a small flattened bug (1–3 mm long) that feeds on *Eucalyptus* and some *Corymbia* species [2, 3]. It employs a lacerate-and-flush feeding strategy [4], causing the loss of photosynthetic surface area, defoliation, and even tree death [2]. This insect is native from Australia, and little research had been done on it until it became a pest of planted *Eucalyptus* trees in Sidney,

in 2002 [5, 6]. It was first recorded outside its natural range in South Africa in 2003, although it was misidentified as *T. australicus* [2]. Originating seemingly from independent introductions from Australia [7], it was first recorded in Argentina in 2005 [6], and it was recognized as the new species *T. peregrinus* [8]. It is now well established in Argentina, Brazil, Uruguay, Chile, and Paraguay [3, 9], and it is foreseen that it will have an important impact for *Eucalyptus* plantations in the region.

Information on the behavior and natural history of *T. peregrinus* is scarce. Our own observations of mating in captivity suggest short precopulatory times after adult emergence (G. Martínez, unpublished), and preoviposition times ranging from 7 to 10 days were recorded in Australia at 20°C [10].

Circumstantial observations suggest that males and nymphs tend to aggregate, possibly by means of semiochemicals. To begin unveiling the possibility of chemical communication in the bronze bug, we conducted a study that comprises twice a week five-instar nymphs emitted from virgin males and females, and Y-tube olfactometer bioassays to test for volatile-based intraspecific attraction. Specifically, we show that males produce a specific volatile compound which we characterized and synthesized, and that this compound acts as a male aggregation pheromone, attracting conspecific males.

2. Materials and Methods

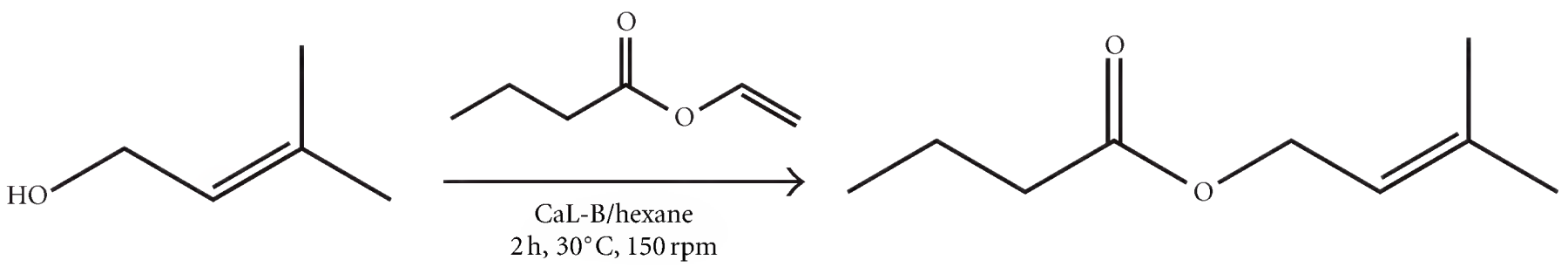
2.1. Insects. Virgin adult bugs were obtained from a laboratory colony reared on *E. tereticornis* (adapted from [10]). Males, females, and nymphs were kept together in mesh-covered cages (35 × 50 × 70 cm) in a greenhouse, on *E. tereticornis* potted plants, and with periodical introduction of field insects. From this stock colony, adults were periodically transferred to *E. tereticornis* branches in Erlenmeyer flasks with distilled water and kept in the laboratory under controlled conditions (20 ± 5°C, 55% RH). Egg clusters harvested from these adult cages were incubated in Petri dishes in a rearing chamber (25°C, 55% RH, 12 : 12 L : D) on leaf discs floating on distilled water. Hatching nymphs were transferred to maturation cages equal to those for adults, and twice a week-five instar nymphs were separated and checked once a day for adult emergence. Just-moulted adults were recognized by the unsclerotized cuticle and were immediately sexed and kept separate for the chemical and behavioral studies. They were kept with *ad libitum* access to *E. camaldulensis* leaves and water.

2.2. Volatile Collection and Analysis. Volatile organic compounds were obtained from virgin males and females enclosed in glass chambers (24 cm length, 4.6 cm diam.) with four *E. tereticornis* leaves. Volatiles from 20 to 40 males and females were adsorbed on 50 mg of Haysep-Q 80/100 mesh, with a current of charcoal-filtered humidified air (300 mL/min) during 72 h (24°C, 14 : 10 D : L photoperiod). The volatiles were eluted with 1 mL distilled hexane and concentrated to 100 μL for GC-MS analysis under a stream of Nitrogen. Volatiles from four *E. tereticornis* leaves were collected in the same fashion as a control. Male volatile extracts for bioassays were obtained similarly (from 29 males), except that only a portion of this extract (200 μL) was concentrated for GC-MS analysis, while most of it was used for bioassays without concentration.

GC-MS analyses were done using a QP-2010 Shimadzu GC-MS, equipped either with a polar (AT-WAX MS) or an apolar (AT-5 MS) column (Alltech) (30 m × 0.25 mm, 0.25 μm), and operated with a constant carrier flow of 1 mL/min (He). The temperature of the GC oven was programmed as follows: for the polar column, the initial temperature was 40°C (1 min), then raised to 250°C at 7°C/min, and held for 1 min at 250°C. For the apolar column, the initial temperature was 40°C (1 min), then raised to 300°C at 10°C/min, and held for 3 min. The injector temperature was 220°C and the interphase temperature 250°C. Injection (1 μL) was in the splitless mode, and mass spectra were acquired from *m/z* 30 to 350 (70 eV, scan mode). For retention index calculations, a mixture of n-alkanes (100 ppm each, in hexane) was injected in the splitless mode immediately after the samples.

2.3. Behavioral Bioassays. All experiments were performed during the day, using a glass Y-tube olfactometer (each arm 20 cm length, 4 cm diam.) with the stimuli placed in separate glass tubes (10 cm length, 4 cm diam.) and connected to the olfactometer by teflon tubing. The relative position of the tested stimulus and its corresponding control were alternated between replicates to prevent any positional bias in the behavior of the insects. Charcoal-filtered humidified air was pushed and pulled through the olfactometer at a total flow of 1200 mL/min. Tested insects were individually placed at the entrance of the central tube, and their behavior was observed for 10 min. First arm choice and time of residence in each arm were recorded, and the results were analyzed using the Chi-square and Wilcoxon tests, respectively. All tested insects were virgin adults and were used only once, and those that did not reach any of the olfactometer arms were not considered in the analysis. Tested stimuli were the following: (a) 10 live males (virgin, with two *E. tereticornis* leaves) versus two *E. tereticornis* leaves; (b) male volatile extracts versus hexane (5 μL, on filter paper, with two *E. tereticornis* leaves); (c) 3-methylbut-2-enyl butanoate versus hexane (1 μg in 5 μL, on filter paper, with two *E. tereticornis* leaves).

2.4. Synthesis. 3-Methylbut-2-enyl butanoate was synthesized from 3-methyl-2-buten-1-ol and vinyl butyrate (Scheme 1), using a biocatalyzed transesterification [11]. Lipase B from *Candida antarctica* (CaL B, 30 mg, Novozym 435) was added to a mixture of vinyl butyrate (0.15 g, 1.3 mmol) and 3-methyl-2-buten-1-ol (0.10 g, 1.2 mmol) in 2 mL of hexane. The mixture was stirred 2 h in an orbital shaker at 30°C. The enzyme was filtered, the solvent was distilled under reduced pressure, and the crude was purified by column chromatography (Hex:AcOEt 8 : 2). 3-Methylbut-2-enyl butanoate was obtained in 98% yield, and its was structure confirmed by NMR and mass spectrometry: ^{1}H NMR (400 MHz, $CDCl_3$): δ(ppm) = 0.94 (t, J = 7.4 Hz, 3H); 1.65 (sext, J = 7.4 Hz, 2H); 1.71 (s, 3H); 1.76 (s, 3H); 2.28 (t, 3H, J = 7.4 Hz); 4.57



3-methyl-2-butenol

3-methyl-2-butenyl butyrate

Scheme 1: Biocatalytic synthesis of 3-methylbut-2-enyl butanoate from 3-methyl-2-buten-1-ol and vinyl butyrate.

(d, J = 7.2 Hz); 5.33 (tsept, J_1 = 7.2 Hz, J_2 = 1.3 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) = 13.6; 17.9; 18.5; 25.7; 36.2; 61.1; 118.7; 138,9; 173.7. MS (IE; 70 eV): m/z = 157 (0.2%), 156 (2.1%), 128 (0.4%), 114 (1.9%), 96 (0.2%), 89 (2.7%), 86 (1.6%), 85 (7.4%), 83 (1.1%), 72 (4.6%), 71 (100%), 70 (4.7%), 69 (62.0%), 68 (89.3%), 67 (56.6%), 66 (1.3%), 65 (1.2%), 57 (2.6%), 56 (1.1%), 55 (3.6%), 54 (1.6%), 53 (14.3%), 51 (1.2%), 44 (2.4%), 43 (63%), 42 (6.5%), 41 (49.8%), 40 (5.1%), 39 (11.4%), 38 (0.6%), 31 (0.4%).

Commercial reactants were purchased from Sigma-Aldrich Inc., and Lipase B from *C. antarctica* (CaL B, Novozym 435) was obtained from Novozymes. Column chromatography was performed using silica gel flash (Kieselgel 60, EM reagent, 230–240 mesh) from Macherey-Nagel. NMR spectra (^{1}H and ^{13}C) were carried out in a Bruker Avance DPX 400 MHz equipment. All experiments were taken at 30°C; $CDCl_3$ was used as solvent and TMS as internal standard [abbreviations: sept(septet); sext (sextet); t(triplet); d(doblet); s(singlet)]. GC-MS analysis was performed as previously described for the volatile extracts.

3. Results

3.1. Volatile Analysis. Several volatile extracts were obtained from equal numbers of males and females, and their GC-MS comparative analyses consistently showed a male-specific compound (Figure 1). This compound eluted with a retention time of 10.1 min in the polar column (RI = 1379) and 7.4 min in the apolar column (RI = 1101). A coeluting compound in the polar column was present in the female volatile extracts and was clearly different from the male compound (Figure 1(b)). This compound was identified as nonanal from its mass spectrum and by comparison with a standard, and it was also found in the leaf volatile extracts (data not shown).

The mass spectrum of the male-specific compound (Figure 1(a)) showed a small molecular ion (2%) at m/z 156 and an M+1 ion of about 10%, suggesting 9 carbon atoms and a possible molecular formula of $C_9H_{16}O_2$. The base peak of m/z 71 suggested a fragment ion with a formula C_4H_7O, as expected from the α-cleavage of a butyric acid ester. In addition, the ion cluster at m/z 67, 68, and 69 is commonly found with varying relative intensities in prenyl esters. In accordance with this, the database search (NIST 08) for this mass spectrum suggested the ester 3-methylbut-2-enyl butanoate (similarity index 96%), which was, therefore, synthesized and compared by GC-MS with the male volatile extracts, resulting in identical retention times and mass spectra between the synthetic and natural compounds (Figure 2).

3.2. Behavioral Bioassays. When live *T. peregrinus* males were used as stimuli in the Y-tube olfactometer, males showed a significant preference toward conspecific males. The olfactometer arm bearing male volatiles was chosen more often (first arm choice: P = 0.004, χ^2 test, N = 35), and the males spent more time in this arm than in the control arm with *E. tereticornis* leaves (P = 0.002, Wilcoxon test, N = 35). The females showed no preference for either olfactometer arm, both regarding first arm choice and residence time in each arm (P = 0.22, χ^2 test, N = 42; P = 0.69, Wilcoxon test, N = 42, resp.) (Figure 3(a)).

The remaining behavioral experiments focused only in the response of males and in evaluating the chemical nature of the male attraction toward conspecific males. It is worth to note, nonetheless, that males did not show any preference in the Y-tube olfactometer in the presence of female volatile extracts as stimulus (first arm choice; stimulus = 27 males; control = 27 males). On the contrary, when male volatile extracts were used as stimulus, the males showed a clear attraction to male volatiles (P = 0.005, χ^2 test, N = 43; P = 0.02, Wilcoxon test, N = 43) (Figure 3(b)). When the results were separated according to male age, the results suggested that the attraction was more important for older males (two-week-old) than for those tested within one week after emergence (P = 0.02, χ^2 test, N = 21; P = 0.09, Wilcoxon test, N = 21; one-week-old males: P = 0.66, χ^2 test, N = 22; P = 0.24, Wilcoxon test, N = 22) (Figure 3(b)). The later, however, were significantly attracted to the synthetic male-specific compound (see below), indicating that they can respond to male odors as well as older males.

Synthetic 3-methylbut-2-enyl butanoate was also attractive to *T. peregrinus* males in Y-tube olfactometer tests. Tested in combination with *E. tereticornis* leaves, 1 μg of the male-specific compound absorbed in filter paper resulted in more males choosing the arm with the stimulus than the control arm ($P < 0.0001$, χ^2 test, N = 88; $P < 0.001$ Wilcoxon test, N = 88) (Figure 3(c)). Differently from the experiments with male volatile extracts, both older and just-emerged males showed a significant attraction toward the synthetic compound (two-week-old males: $P < 0.0001$, χ^2 test, N = 42; $P < 0.02$, Wilcoxon test, N = 42; one-week-old males: $P < 0.001$, χ^2 test, N = 46; P = 0.002, Wilcoxon test, N = 46) (Figure 3(c)).

Considering the number of males used for the male

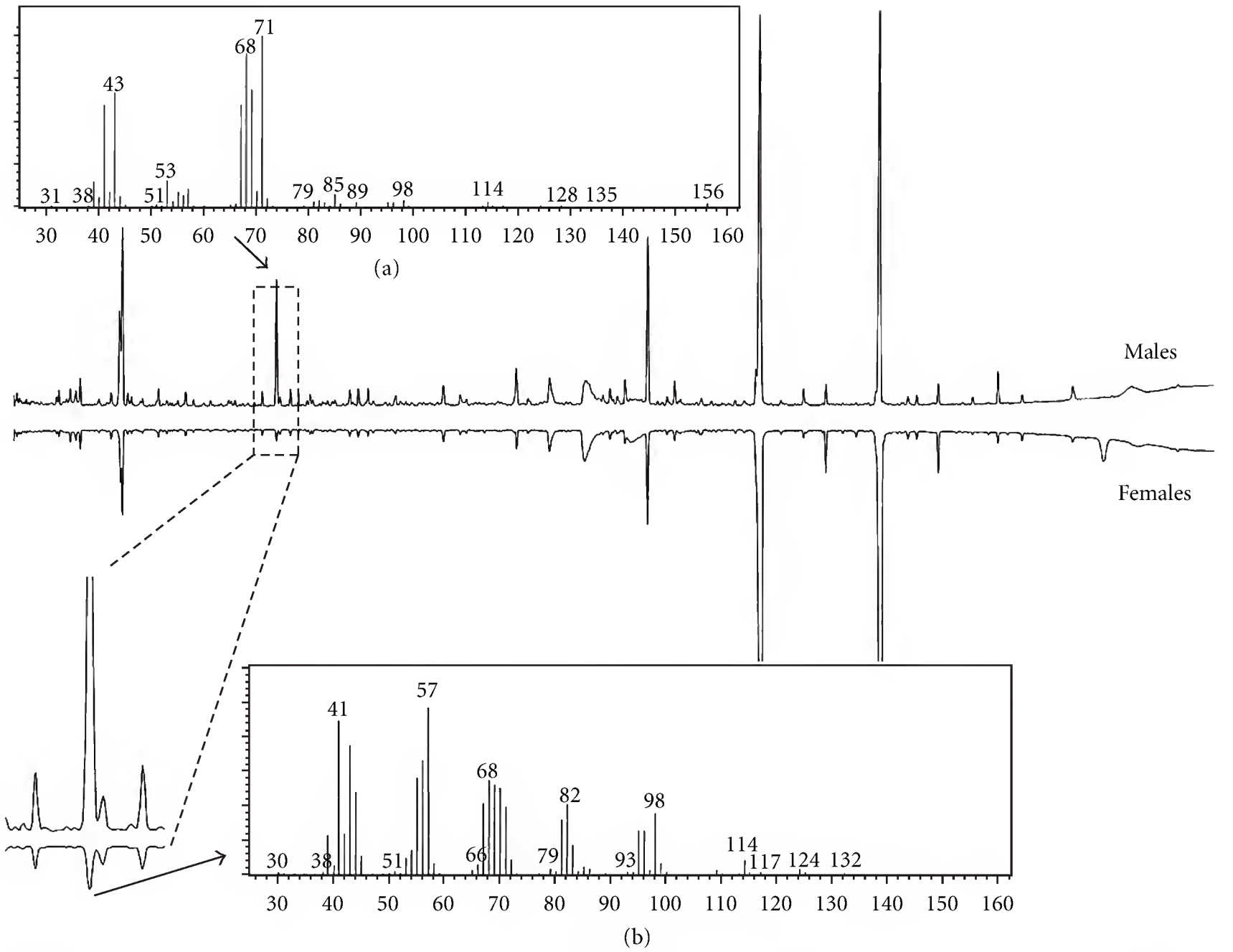


Figure 1: GC-MS traces (TIC) of *T. peregrinus* male (top) and female (bottom) volatile extracts analyzed in a polar column. A male-specific compound was present in male volatile extracts, with a retention time of 10.1 min (mass spectrum shown in insert (a)). A coeluting compound in female volatile extracts was identified as nonanal based on its mass spectrum (insert (b)) and comparison with a synthetic standard. This compound was also present in *E. tereticornis* leaf volatiles.

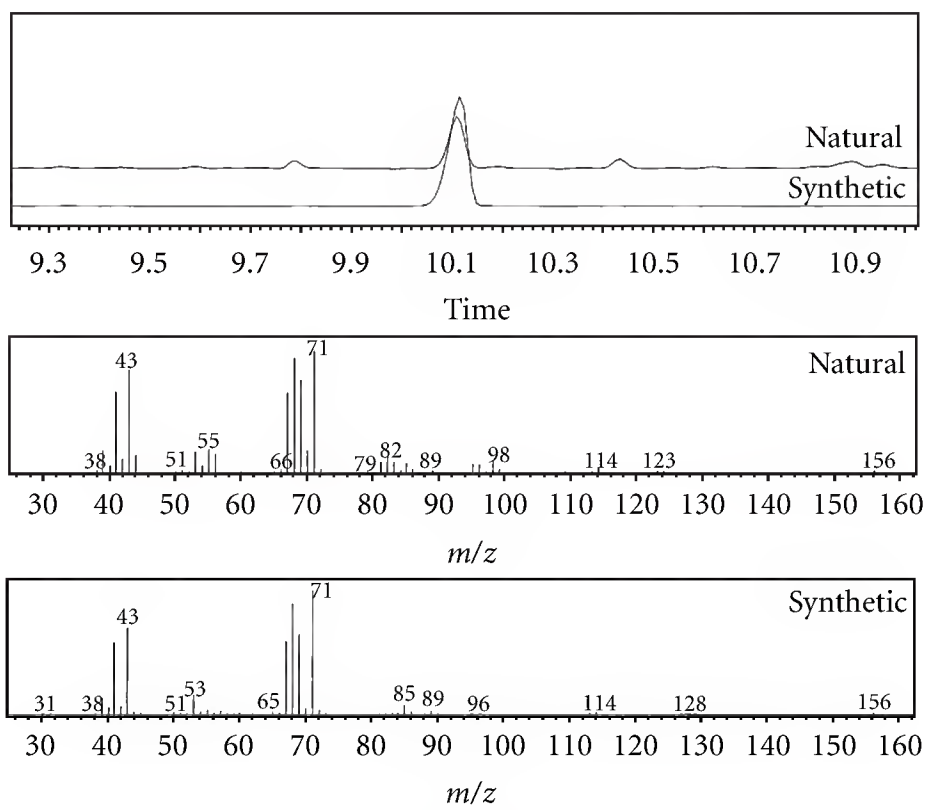


Figure 2: GC-MS traces (TIC) of *T. peregrinus* male volatile extracts (upper trace) and synthetic 3-methylbut-2-enyl butanoate (lower trace). The mass spectra of the natural and synthetic compounds match closely, with small ions in the natural sample corresponding to nonanal, a coeluting compound from *E. tereticornis* leaf volatiles.

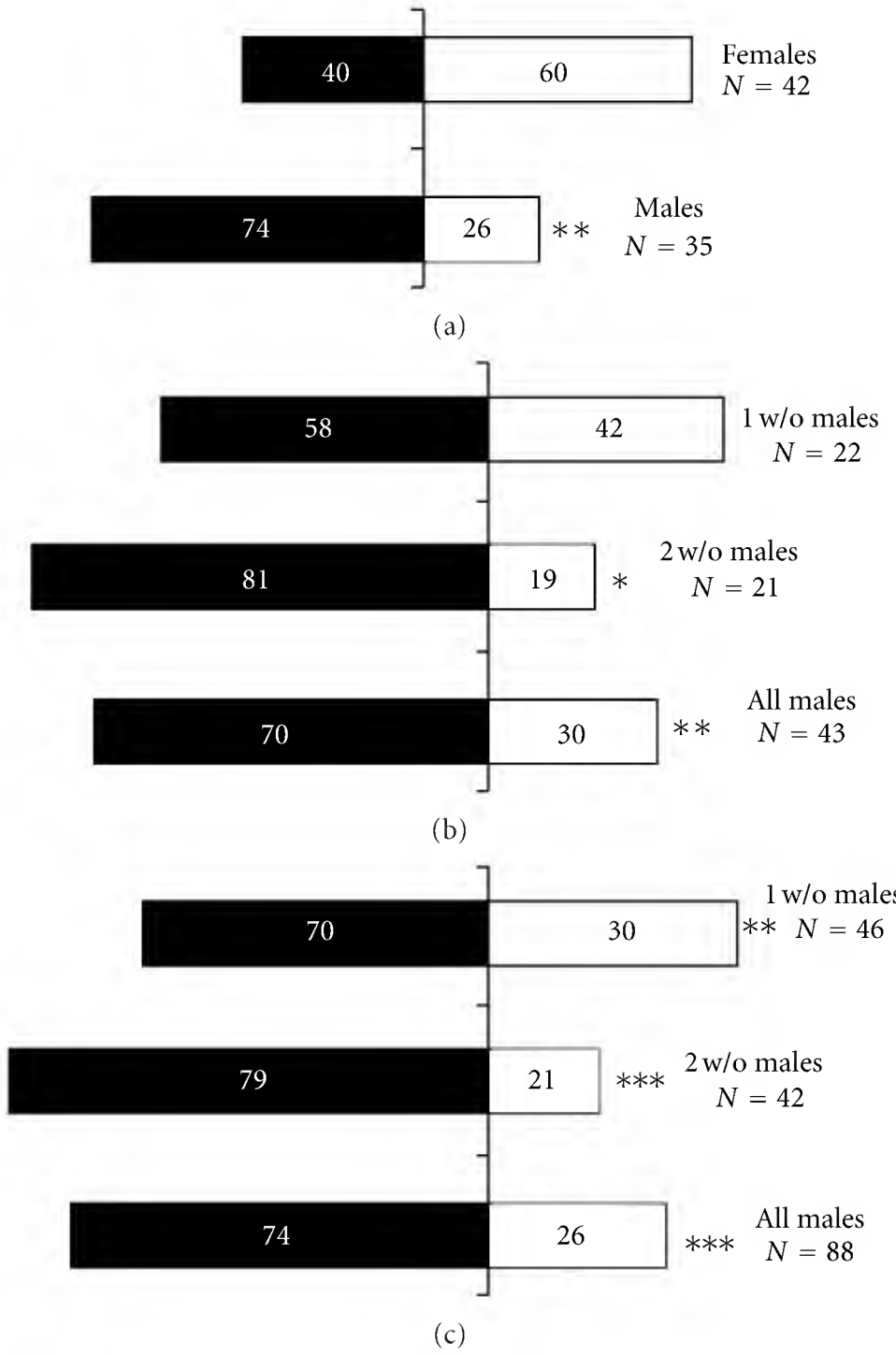


FIGURE 3: First arm choice of *T. peregrinus* adults in Y-tube olfactometer tests. Bars show the percent (numbers within bars) of insects choosing the stimulus arm (black bars) or the control arm (white bars). (a) Response of females and males to volatiles from live males versus control. (b) Response of one- and two-week-old (w/o) males to male volatile extracts versus hexane. (c) Response of one- and two-week-old males to synthetic 3-methylbut-2-enyl butanoate versus hexane. All treatments and controls included 2 leaves of *E. tereticornis*. Asterisks indicate significance levels in Chi-square tests ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

volatile collections, and the time of collection, bioassays of the male volatile extracts were performed with 0.002 $\text{insect}_{\text{eq}} \cdot \text{h}^{-1}$. In addition, by comparing the peak areas of 3-methylbut-2-enyl butanoate in the tested extracts and in a 100 ppm solution of the synthetic compound, it can be estimated that the amount of 3-methylbut-2-enyl butanoate in 5 μL of the tested extract was 0.25 μg. The amount of synthetic material used in the bioassays was, therefore, larger, but in the same order of the estimated amount in the bioassays with the extracts. Moreover, in a parallel study, Martins et al. (2012, this issue) report that whole body extracts of *T. peregrinus* males contain up to 1 μg/insect of 3-methylbut-2-enyl butanoate, indicating that the amounts used in our bioassays are biologically relevant.

4. Discussion

To our knowledge, this is the first report of 3-methylbut-2-enyl butanoate as an insect semiochemical, and the first pheromone described in the small Thaumastocoridae family. The same compound is being reported in a simultaneous and independent study by Martins et al. (2012, this issue), confirming its occurrence in different populations of *T. peregrinus* in South America. These authors also found a small amount of the compound in female extracts, and another minor male-specific compound in male extracts, both of which we did not find probably due to differences in the sampling procedure (whole body extracts versus volatile extracts).

Short-chain aliphatic esters are common pheromone components in true bugs [12]. A positional isomer of 3-methylbut-2-enyl butanoate, (*E*)-2-methylbut-2-enyl butanoate, has been reported as a female-specific, male-attractant pheromone component in the broad-headed bug *Alydus eurinus* (Alydidae). Males, and to a lesser extent females and nymphs, were attracted to blends containing this and other butyrate esters produced in the metathoracic glands [13]. Aliphatic butyrate and hexanoate esters are also common pheromones in the Miridae family [12], which shares the superfamily Miroidea with the Thaumastocoridae [14]. The alcohol moieties of these esters are, however, clearly not of terpenic origin, which is most likely the case with the 3-methylbut-2-enyl portion of the male compound in *T. peregrinus*.

Different from the pentatomids, in which most sex or aggregation pheromones are emitted by the males, the few species for which pheromones have been identified in the closely related Miridae use sex pheromones produced by the females, or compounds emitted by both sexes but to which only males are attracted [12, 15–17]. Other than sex pheromones, a male-produced anti-sex pheromone (or male repellant), which has remarkably the same chemical *motiv* and anatomical origin of female phermones, has been reported in two mirid species, suggesting a mate-guarding strategy in these and possibly other species in the family [18, 19]. Our behavioral studies with *T. peregrinus* did not show any cross-gender attraction mediated by sex pheromones, but rather a volatile-mediated male attraction toward conspecific males. Although such male-male chemical interaction does not strictly fit the commonly used definition of an aggregation pheromone (both sexes attracted), we consider that the male-specific compound herein reported, 3-methylbut-2-enyl butanoate, can be regarded as a male aggregation pheromone, or pheromone component. Indeed, our results show that males were attracted to (a) odors from live males in Y-olfactometer bioassays (Figures 3(a) and 4(a)), (b) male volatile extracts (Figures 3(b) and 4(b)), and (c) synthetic 3-methylbut-2-enyl butanoate (Figures 3(c) and 4(c)). Of note is that all our behavioral experiments included *E. tereticornis* leaves in both arms of the olfactometer. We tested the attraction toward live insects with leaves to prevent the insects from desiccation; therefore, tests with volatile extracts and synthetic 3-methylbut-2-enyl butanoate were conducted similarly, in order to compare the results from the different

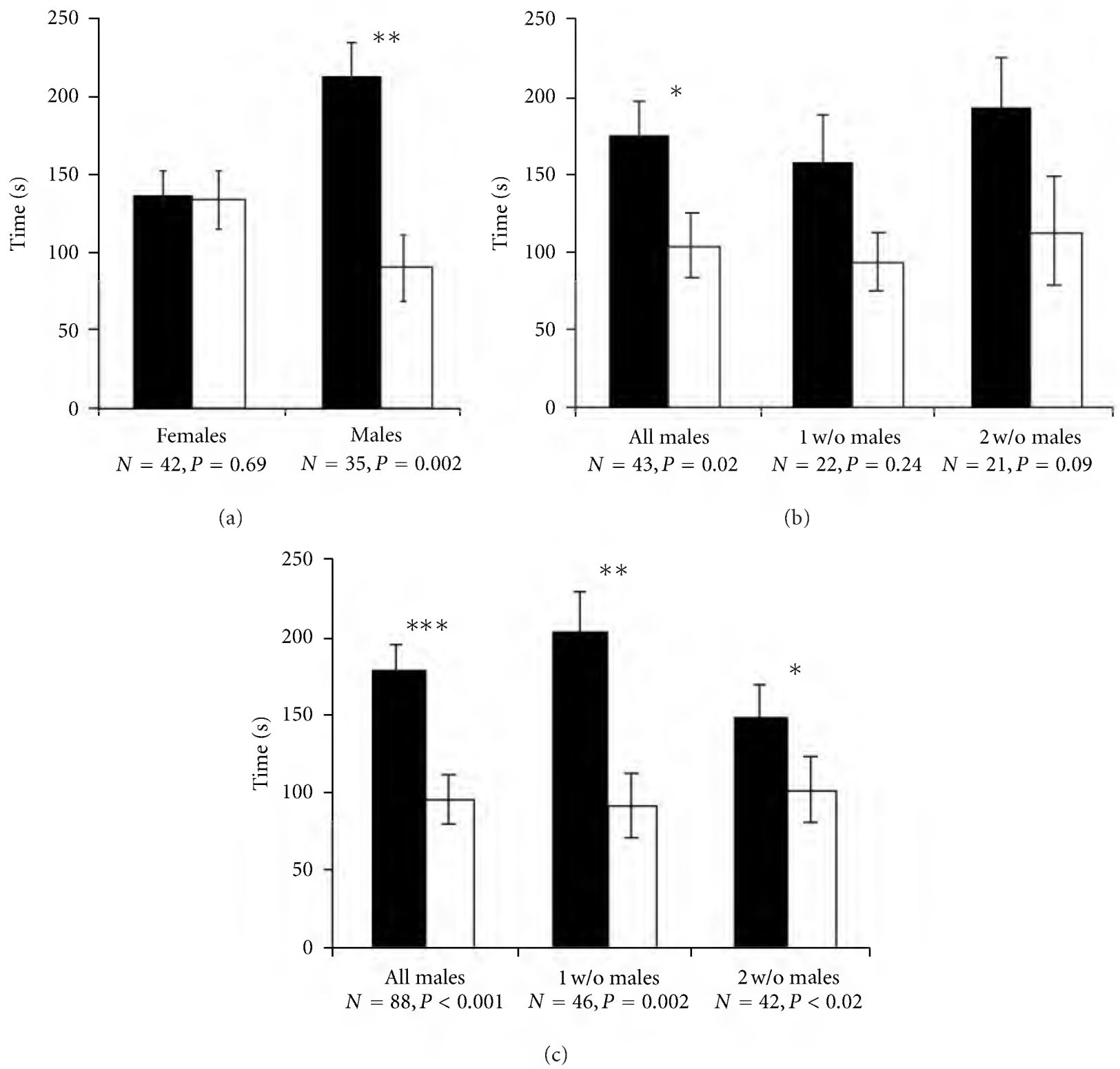


FIGURE 4: Time of permanence of *T. peregrinus* adults in the stimulus and control arms of the Y-tube olfactometer. Bars show the cumulative time (in 10 min.) that the insects spent in the stimulus arm (black bars) or the control arm (white bars). (a) Response of females and males to volatiles from live males versus control. (b) Response of one- and two-week-old (w/o) males to male volatile extracts versus hexane. (c) Response of one- and two-week-old males to synthetic 3-methylbut-2-enyl butanoate versus hexane. All treatments and controls included 2 leaves of *E. tereticornis*. Asterisks indicate significance level in Chi-square tests ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

experiments. Although attraction to host plant odors cannot be ruled out from our results, the use of leaves in both olfactometer arms throughout our study allowed for an independent test of the added stimuli. It remains to be studied, nonetheless, if host plant volatiles play an additional, possibly synergic role in the attraction of males.

Finally, our olfactometer tests with male volatile extracts suggest an age-dependant difference in male attraction, since one-week-old males did not show a significant preference (Figure 3(b)). However, the trend in younger males was similar to that of older males, and one-week-old males clearly responded to synthetic 3-methylbut-2-enyl butanoate (Figure 3(c)). Therefore, a possible effect of age in the response of males needs further investigation. Interestingly though, chemical data published simultaneously to our study show that older males produce more 3-methylbut-2-enyl butanoate than younger ones (Martins et al., 2012, this issue), and one can speculate that a correlation between production of and response to this male aggregation pheromone may occur. The ecological significance of this aggregation pheromone, and its possible application for the management of *T. peregrinus* in *Eucalyptus* commercial plantations, will be further investigated.

Acknowledgments

The authors wish to acknowledge grants by the National Agency of Research and Innovation (ANII: PR_FSA-2009-1-1522) and partial funding by the National Institute of Agricultural Research (INIA: Grant FO6).

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 940962, 10 pages
doi:10.1155/2012/940962

Research Article

Bark Beetles, *Pityogenes bidentatus*, Orienting to Aggregation Pheromone Avoid Conifer Monoterpene Odors When Flying but Not When Walking

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Received 17 December 2011; Revised 14 March 2012; Accepted 25 April 2012

Academic Editor: Brian T. Sullivan

Previous studies and data presented here suggest that odors from healthy host Scotch pine (*Pinus sylvestris*) and nonhost Norway spruce (*Picea abies*), as well as major monoterpenes of these trees at natural release rates, significantly reduce the attraction of flying bark beetles, *Pityogenes bidentatus*, of both sexes to their aggregation pheromone components grandisol and *cis*-verbenol in the field, as tested by slow rotation of trap pairs. In contrast, *P. bidentatus* males and females walking in an open-arena olfactometer in the laboratory did not avoid monoterpene vapors at release rates spanning several orders of magnitude in combination with aggregation pheromone. The bark beetle may avoid monoterpenes when flying as a mechanism for avoiding nonhost species, vigorous and thus unsuitable host trees, as well as harmful resinous areas of hosts. Inhibition of this flight avoidance response in beetles after landing would allow them to initiate, or to find and enter, gallery holes with high monoterpene vapor concentrations in order to feed and reproduce.

1. Introduction

The bark beetle *Pityogenes bidentatus* (Herbst) (Coleoptera: Curculionidae: Scolytinae) is a relatively small (2-3 mm long) insect that attacks only Scotch pine, *Pinus sylvestris* L. [1]. The beetle is common in Scotch pine and mixed conifer forests of Europe where it begins a seasonal flight in spring and colonizes smaller diameter trunks and limbs of weakened hosts [1–3]. The species builds up in slash and logging residue and can harm adjacent young stands of Scotch pine [3]. *P. bidentatus* have been caught more frequently in traps baited with pine logs left for several weeks compared to freshly-cut logs, indicating that the beetle is attracted to odors from aging tissue [4]. The aggregation pheromone of *P. bidentatus* consists of two components, (*S*)-*cis*-verbenol (cV) and grandisol (G1) as determined in previous studies [5–9]. Grandisol is well known as a pheromone component of the boll weevil, *Anthonomus grandis* Boheman [10].

Injured conifers such as pines and spruce produce resin, commonly consisting of about 80% mildly toxic monoterpenes, to defend against the penetrations of attacking insects [11]. Some species of bark beetles may be attracted to these tree-specific blends of monoterpenes [12, 13] since they indicate both the appropriate host and the likely susceptibility to colonization [14–17]. Bark beetles in the genus *Tomicus* exhibit relatively strong attraction to host Scotch pine and its major monoterpenes, especially α-pinene (both enantiomers), (+)-3-carene, and terpinolene [14–17]. Camphene, (+)- and (−)-α-pinene, and (−)-β-pinene are major monoterpenes of Norway spruce, *Picea abies* L., the host of *P. chalcographus* L. These monoterpenes enhance the attraction of flying *P. chalcographus* to traps baited with its aggregation pheromone, and increase entry rates of the beetles through 2.5 mm diameter holes into the traps [18, 19]. Furthermore, several studies have found that certain monoterpenes enhance attraction to pheromone components in some of the more aggressive bark beetles that kill standing trees [18–25].

On the other hand, a sufficient flow of resin can expel or kill attacking bark beetles. Hence less aggressive species of bark beetles that specialize on hosts with compromised resin defenses may have evolved olfactory mechanisms and

behaviors for the avoidance of specific volatile monoterpenes in tree resins indicative of a vigorous and resistant tree. Likewise beetles apparently avoid certain monoterpenes or other volatile chemicals associated specifically with nonhost tree species [26–37]. This appears to be the case for flying *P. bidentatus* when orienting to their aggregation pheromone components. Odors from freshly-cut host Scotch pine or from non-host Norway spruce, as well as non-host deciduous trees (birch, *Betula pendula* Roth.; mountain ash, *Sorbus aucuparia* L.; oak, *Quercus robur* L. and alder buckthorn, *Frangula alnus* P. Mill.) reduced attraction to the aggregation pheromone components [7, 8]. Many individual monoterpenes and blends released at rates comparable to that released from physical wounds of trees also inhibited flight attraction to their aggregation pheromone [7–9].

Three previous studies [7–9] tested effects of monoterpenes on flying *P. bidentatus* by using a pair of traps separated 6 m apart that were mechanically rotated slowly at 2 rph to even out any trap position effects [7, 8, 38]. Both traps contained aggregation pheromone (G1 and cV), while one trap also released host or nonhost odors (specific monoterpenes, cut bark, or twigs) that reduced attraction of flying *P. bidentatus* (Figure 1). The objective of my study was to assess the response of both flying and walking *P. bidentatus* of both sexes to monoterpenes released in association with the aggregation pheromone (assayed with either rotating traps in the field or a laboratory olfactometer, resp.). The hypothesis was that aggregation pheromone responses by beetles walking in the olfactometer would exhibit the same inhibition to monoterpenes as when flying in the field. This hypothesis was based on earlier studies with bark beetle semiochemicals in which the same behavioral responses (attraction or repulsion) were found both when beetles were walking in a laboratory olfactometer and when flying in the field to baited traps [7, 8, 14, 15, 18, 19, 39–42].

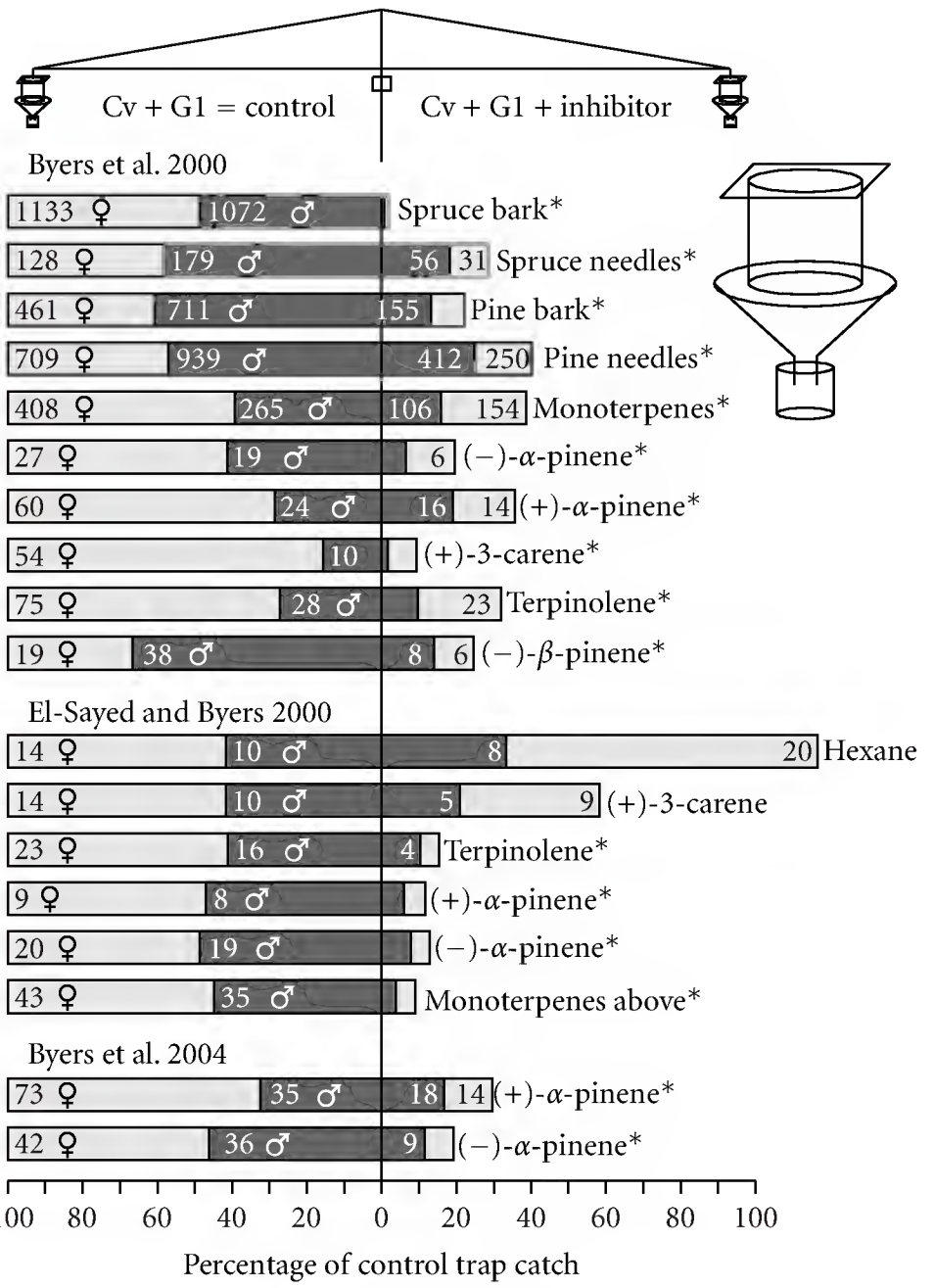

Figure 1: Selected comparisons from three studies (Byers et al. [7], El-Sayed and Byers [9], and Byers et al. [8]) showing reduced catches of flying *Pityogenes bidentatus* on traps releasing pheromone plus inhibitor volatiles (monoterpenes released at 1 mg/day, 100–200 g bark or needles of spruce and pine) compared to control traps with pheromone alone (Sjöbo/Veberöd, Sweden, April-May 1998–2000, see Methods for details). Total catches of both sexes with asterisks were significantly lower than the pheromone controls in the same test at $P < 0.01$ (chi-square goodness of fit).

2. Materials and Methods

2.1. Inhibition of Attractive Response of Flying P. bidentatus to Aggregation Pheromone Components by Monoterpenes. Field tests similar to those mentioned above [7–9] were conducted in mixed forests/plantations (primarily Scotch pine) near Sjöbo/Veberöd, Sweden in May 2001 with three sets of rotating trap pairs. Traps in each pair were 6 m apart, suspended at 1.2 m height, and rotated around a central axis at 2 rph. Synthetic aggregation components G1 (racemic, *cis*-grandisol, (1*R*, 2*S*)-1-(2-hydroxyethyl)-1-methyl-2-(1-methylethenyl) cyclobutane, >98%, Frank Enterprises, Inc., Columbus, Ohio) and cV ((1*S*,4*S*,5*S*)-*cis*-verbenol, 96%, Borregaard) were placed inside each trap in a pair. Each pheromone bait had 25 μL G1 at the bottom of a small glass tube (34 mm × 4.45 mm i.d. opening) and ~25 mg of crystalline cV at the bottom of a polyethylene tube (31 mm × 6.15 mm i.d. opening) so that releases were nearly constant (0.05 mg G1/day and 0.5 mg cV/day at 22°C).

Each trap consisted of an 18 cm diameter × 28 cm high transparent polycarbonate cylinder covered at the top but open at the bottom and suspended over a large white plastic funnel (31 cm diameter) that collected beetles striking the cylinder (Figure 1). Experimental runs of each rotating trap pair were conducted for at least 1 hour during 11 : 00–18 : 00 when temperature was above 18°C. After each run the inhibitory source, but not the attractants, was switched to the other trap of the pair such that from two to five runs were conducted for each monoterpene test. The monoterpenes tested as inhibitors included (+)-α-pinene ($[\alpha]_D^{20} = +57°$, 99%, Fluka, Stockholm, Sweden), (−)-α-pinene ($[\alpha]_D^{20} = -50°$, >99.5%, Fluka), (+)-3-carene ($[\alpha]_D^{20} = +15°$, 95%, Aldrich, Stockholm, Sweden), terpinolene (97% Carl Roth Gmbh, Karlsruhe, Germany), and (−)-β-pinene ($[\alpha]_D^{20} = -21°$, 99%, Aldrich), myrcene (95%, Aldrich), (−)-limonene ($[\alpha]_D^{20} = -94°$, 96%, Aldrich), and (+)-limonene ($[\alpha]_D^{20} = +123°$, 97%, Aldrich). The release rates (μg/h) were about 1400 for α-pinene enantiomers, 800 for (−)-β-pinene, 740 for myrcene, 385 for each enantiomer of limonene, and 240 for terpinolene released individually from glass tubes as described above (weight loss at 22°C in laboratory).

The catches on inhibitor and control were pooled for each treatment comparison and tested for significant differences by comparing the two catches to the average catch with a chi-square test ($df = 1$). If the tests were statistically significant ($P < 0.05$), then catches of each sex were compared within a treatment comparison for significant differences using a chi-square test. Previous results (Figure 1) [7, 8] were also analyzed for gender differences in regard to inhibition of attraction.

Previously reported dose-response data for monoterpene reduction of *P. bidentatus* attraction to G1 and cV (Figure 4 in [9]) was subjected to user-defined logarithmic and logistic dose-response regressions (Statistica 5.1, StatsSoft Inc., Tulsa OK) to find a better relationship than originally presented. In this previously reported test, responses of both sexes to the pheromone-baited, slow-rotating trap pairs were compared after one trap of the pair was amended with an exact release of four host monoterpenes ranging from 0.01 to 10 Scotch pine log equivalents (0.1 to 100 μg/min each monoterpene) provided by a piezoelectric sprayer and syringe pump [9].

2.2. Tests for Inhibition of the Attractive Response of Walking P. bidentatus to Aggregation Pheromone Components in a Laboratory Open-Arena Olfactometer. Adults of *P. bidentatus* that had been caught live in traps baited with aggregation pheromone components, G1 and cV at the same field sites described above during May 1999 and 2000 were separated by sex in the laboratory. They were then stored at 4°C on moistened tissue paper in Petri dishes for up to four days until use in bioassays. The responses of *P. bidentatus* to semiochemicals were tested in a modified open-arena olfactometer [14, 15, 19, 41, 43]. This consisted of a suction fan that drew air out of the room through a fine metal screen (0.4 mm mesh) on one side of an opaque plastic manifold (60 cm wide × 25 cm × 25 cm) placed on the "downwind" side of a glass tabletop covered with white construction/poster paper (0.84 × 1.1 m). At the "upwind" end of the table (0.7 m from the manifold), laboratory air was forced through a clear acrylic manifold (46 cm wide × 5 cm high × 8.5 cm deep) with three rows of 13, 12, and 13 holes (1.5 mm diameter) starting 7.5 mm above the surface (with spacing between holes 3 cm horizontally and 1 cm vertically; middle row of 12 holes centered). The two manifolds maintained an approximately laminar airflow with a speed of 0.9 m/s at the semiochemical source (5 cm from the center of upwind manifold) and 0.6 m/s where the beetles were released (21 ± 2 cm "downwind" from the source). Ten beetles of a single sex were released together initially. A positive response was recorded when a beetle arrived within 2 cm of the odor source. Beetles that walked outside a 25 cm radius circle centered on the release point, or that had not reached the odor source in the time required for various mixtures of the semiochemicals in diethyl ether to finish eluting (126 ± 10 sec) from a 5 μL glass capillary (Drummond Scientific, Broomall, PA), were placed temporarily in a plastic Petri dish until the first trial was completed. These nonresponding beetles were released a second time to a newly filled capillary tube and the numbers reaching the odor source in the two trials were summed (e.g., two of ten may have responded in the first trial and two of eight in the second trial giving a 40% response in total).

The aggregation pheromone components cV and G1 were released together in the laboratory in two ways: (1) diluted in diethyl ether and placed in a 5-μL capillary tube open at both ends, or (2) in the field dispensers described above. The release rates for the second method were estimated by weight loss at 22°C to be 350 ng/min for cV and 35 ng/min for G1, the same rates released in the slow-rotating pairs of traps in field tests. The release of monoterpenes was by the first method only, although the compounds were also dispensed neat from the 5-μL capillary. In method 1, release rates of chemicals from the 5-μL capillary were dependent on the evaporation of the solvent diethyl ether; that is, 2.2 μL of ether was released per min and thus the release rates were about 2.2 times the concentration (in mass per μL) for each dilution tested as indicated in Tables 1, 2, and 3.

The monoterpenes tested were (+)-α-pinene, (−)-α-pinene, (+)-3-carene, terpinolene, and (−)-β-pinene as described above. The two aggregation pheromone components (G1 and cV) were tested together without monoterpenes or together with monoterpenes and compared in some cases to a diethyl ether control in three sets of bioassays (4 May 1999, 7 June 1999, and 21–23 May 2000). At least 40 beetles of each sex were tested for each release rate of the compounds under conditions of 22°C and 1700 lux during 13 : 00–17 : 00. Both sexes were tested at the lowest chemical concentrations initially each day and then additional beetles were tested at increasingly higher concentrations as indicated in the tables. The release rates were chosen based on behavioral results with other bark beetles in previous studies with the same olfactometer [14–16, 19] as well as to correspond to rates used in the field (which represent natural release rates from Scotch pine logs or bark beetles) [9, 14–18]. The release of neat monoterpenes in the open-arena bioassay were determined precisely in 2012 under the same olfactometer conditions by measuring changes in meniscus volume of the capillary as monitored by a time-lapse webcam and computer (Byers unpublished). Statistically significant differences in the percent responding between various release rates and compound combinations were determined by a chi-square test.

3. Results

3.1. Inhibition of Attractive Response of Flying P. bidentatus to Aggregation Pheromone Components by Monoterpenes. In tests of rotating pairs of aggregation pheromone-baited traps, the single trap of each pair dispensing vapor of individual monoterpenes generally caught fewer *P. bidentatus* than its paired control trap (Figure 2). Myrcene was not tested in the earlier studies (Figure 1) and was inhibitory to *P. bidentatus* response (Figure 2). Also, the enantiomers of limonene had not been tested earlier and, contrary to other monoterpenes, these did not appear to reduce response of either sex of the bark beetle (Figure 2). In tests that showed a significant reduction in total catch by monoterpenes (Figure 2), attraction of each sex was inhibited in flight in

Table 1: Percent of walking *Pityogenes bidentatus* females and males responding in a laboratory olfactometer (4 May 1999) to conifer monoterpenes and aggregation pheromone components (G1 = grandisol, cV = (*S*)-*cis*-verbenol).

Chemicals[a]	Percent responding[b]	95% B. C. L.[c]	*N*
Females			
Diethyl ether control	10.0a	4.0–23.1	40
A = G1 + cV at 5×10^{-10} g/μL	42.5b	28.5–57.8	40
A + monoterpenes[d] at 5×10^{-9} g/μL	37.5b	24.5–53.0	40
A + monoterpenes[d] at 5×10^{-8} g/μL	30b	18.1–45.4	40
A + monoterpenes[d] at 5×10^{-7} g/μL	32.5b	20.1–48.0	40
A + monoterpenes[d] at 5×10^{-6} g/μL	37.5b	24.2–53.0	40
Females			
B = G1+ cV at 5×10^{-9} g/μL	72.5a	49.5–77.9	40
B + monoterpenes[d] at 5×10^{-7} g/μL	52.5a	37.5–67.1	40
B + monoterpenes[d] at 5×10^{-6} g/μL	57.5a	42.2–71.5	40
Females			
C = G1+ cV field dispensers	45a	34.6–55.9	80
C + monoterpenes[d] at 5×10^{-5} g/μL	48.75a	38.1–59.5	80
Males			
C	33.33a	22.7–45.9	60
C + monoterpenes[d] at 5×10^{-5} g/μL	38.33a	27.1–51.0	60

[a] Chemicals dispensed by evaporation from 5 μL micropipette at indicated concentration each/μL diethyl ether (release rate approximately 2.2 × concentration/min).
[b] Percentages followed by same letter were not significantly different ($\alpha = 0.05$, chi-square) within a test series for each sex.
[c] Binomial confidence limits for proportions.
[d] Major host Scotch pine monoterpenes: (+)-α-pinene, (−)-α-pinene, (+)-3-carene, and terpinolene were dispensed in diethyl ether solution at concentrations indicated in table.

Table 2: Percent of walking *Pityogenes bidentatus* females and males responding in a laboratory olfactometer (7 June 1999) to conifer monoterpenes and aggregation pheromone components (G1 = grandisol, cV = (*S*)-*cis*-verbenol).

Chemicals[a]	Percent responding[b]	95% B. C. L.[c]	*N*
Females			
B = G1+ cV at 10^{-9} g/μL	68.3a	55.8–78.7	60
B + monoterpenes[d] at 10^{-5} g/μL	65.0a	52.4–75.8	60
B + monoterpenes neat[e]	70.0a	57.5–80.1	60
B + (−)-α-pinene neat[e]	75.0a	62.8–84.2	60
Males			
B	65.0a	52.4–75.8	60
B + (−)-α-pinene neat[e]	71.7a	59.2–81.5	60

[a] Chemicals dispensed by evaporation from 5 μL micropipette at indicated concentration each/μL diethyl ether (release rate approximately 2.2 × concentration/min except when neat).
[b] Percentages followed by same letter were not significantly different ($\alpha = 0.05$, chi-square).
[c] Binomial confidence limits for proportions.
[d] Major host Scotch pine monoterpenes: (+)-α-pinene, (−)-α-pinene, (+)-3-carene, and terpinolene were each at indicated concentration as dispensed in diethyl ether solution.
[e] Chemicals were dispensed neat from 5 μL micropipettes; (−)-α-pinene, (+)-3-carene, and terpinolene were each released at 28, 9.4, and 4.8 μg/min, respectively, according to capillary measurements over time in the olfactometer.

nearly all cases ($P < 0.01$), except male response to (−)-α-pinene ($P = 0.02$) and to (+)-α-pinene ($P = 0.012$) were only marginally significant due likely to low numbers. The inhibition of the beetle by (+)-3-carene ($P < 0.01$) was mainly due to females ($P < 0.01$) because male catches were not significantly different ($P = 0.56$), but again the numbers of males caught were low (Figure 2). However, males and females were inhibited in an earlier study [7] by (+)-3-carene (Figure 1, both sexes $P < 0.01$). In the reanalysis of previous studies catches of each gender (Figure 1), both sexes were significantly inhibited in flight by non-host Norway spruce and host Scotch pine odors as well as by several monoterpenes: α-pinene, β-pinene, 3-carene, and terpinolene tested singly or as a combination (all $P < 0.01$).

Table 3: Percent of walking *Pityogenes bidentatus* females and males responding in a laboratory olfactometer (21–23 May 2000) to conifer monoterpenes and its aggregation pheromone components (G1 = grandisol, cV = *S-cis*-verbenol).

Chemicals[a]	Percent responding[b]	95% B. C. L.[c]	*N*
Females			
Diethyl ether control	10.0a	4.0–23.1	40
A = G1 + cV at 5×10^{-10} g/μL	25.0ab	14.2–40.2	40
B = G1 + cV at 5×10^{-9} g/μL	35.0b	22.1–50.5	40
G1 + cV at 5×10^{-8} g/μL	42.5bc	28.5–57.8	40
D = G1 + cV at 5×10^{-7} g/μL	62.5cd	47.0–75.8	40
D + (−)-β-pinene at 10^{-5} g/μL	72.5d	57.2–83.9	40
D + (−)-α-pinene at 10^{-5} g/μL	65.0cd	49.5–77.9	40
Males			
Diethyl ether control	5.0a	1.4–16.5	40
A = G1 + cV at 5×10^{-10} g/μL	17.5a	8.7–32.0	40
B = G1 + cV at 5×10^{-9} g/μL	42.5b	28.5–57.8	40
G1 + cV at 5×10^{-8} g/μL	40.0b	26.3–55.4	40
D = G1 + cV at 5×10^{-7} g/μL	50.0b	35.2–64.8	40
D + (−)-β-pinene at 10^{-5} g/μL	47.5b	32.9–62.5	40
D + (−)-α-pinene at 10^{-5} g/μL	55.0b	39.8–69.3	40

[a] Chemicals dispensed by evaporation from 5 μL micropipette at indicated concentration each/μL diethyl ether (release rate approximately 2.2 × concentration/min).
[b] Percentages followed by same letter were not significantly different ($\alpha = 0.05$, chi-square).
[c] Binomial confidence limits for proportions.

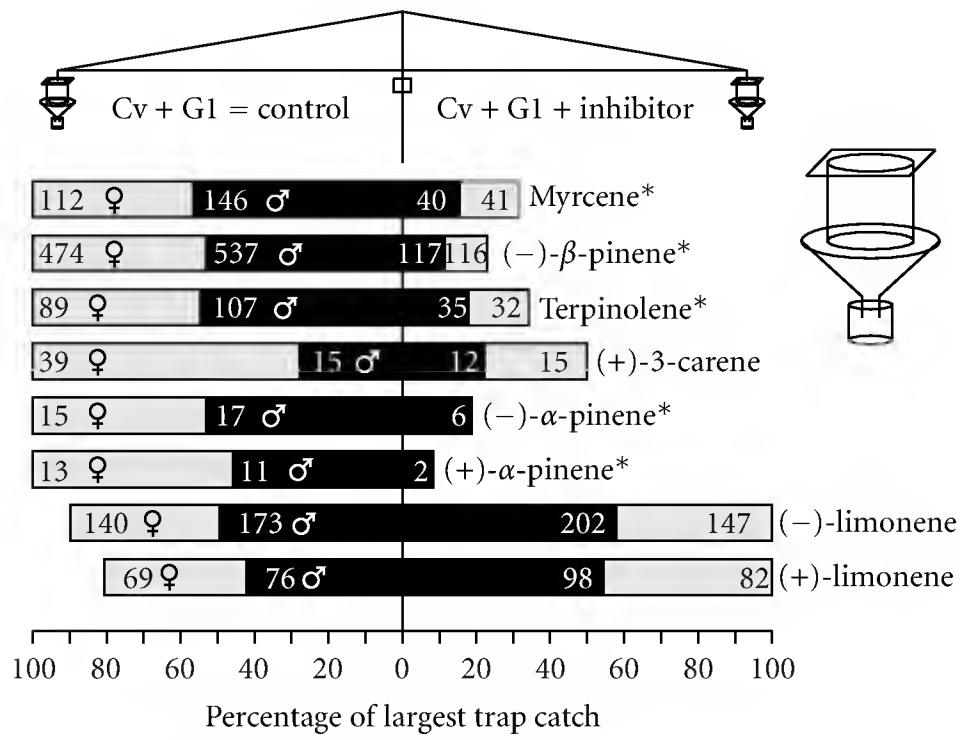

Figure 2: Attraction of flying *Pityogenes bidentatus* to pairs of slowly-rotated traps releasing aggregation pheromone (*cis*-verbenol = Cv and grandisol = G1) when individual monoterpenes were released from one of the two traps. Tests were performed at different dates and times during May 2001 in Sjöbo/Veberöd, Sweden. Numbers inside light and black bars represent catch of each sex. Treatments with asterisks caught significantly fewer beetles (sexes summed) in the treatment than the control trap in the same pair ($P < 0.01$, chi-square test).

In one study [7], however, males were not inhibited by (+)-α-pinene ($P = 0.20$) possibly due to low numbers trapped (10 and 5 males). In a second study [8], both sexes were inhibited by either enantiomer of α-pinene (all $P < 0.01$). In a third study [9], (+)-3-carene was not significantly inhibitory for either sex (both $P > 0.2$), although low numbers were caught as in the second study (Figure 1).

Reanalysis of a similar published study [9], in which one trap of each pair had exact releases of four host monoterpenes representing 0.01 to 10 pine log equivalents, revealed that the dose-response data best fit a logistic regression ($R^2 = 0.86$; equation in Figure 3). The common logarithmic regression, $Y = 24.824 - 5.087 \cdot \ln(X)$, also fits well ($R^2 = 0.84$).

3.2. Tests for Inhibition of the Attractive Response of Walking P. bidentatus to Aggregation Pheromone Components in a Laboratory Open-Arena Olfactometer. The responses of walking female *P. bidentatus* to aggregation pheromone components grandisol (G1) and *cis*-verbenol (cV) at 5×10^{-10} g/μL concentration was 42.5% (Table 1). An increasing dosage of four host Scotch pine monoterpenes (each compound ranged from 10 to 10,000 times the concentration of the pheromone components) did not significantly affect the attraction of walking females to this concentration of G1 and cV (Table 1), as would be expected from the field trapping experiments above. In another bioassay series, a stronger aggregation dose of 5×10^{-9} g/μL caused 72.5% of females to respond, and the addition to this dosage of the four monoterpenes at 1000 to 10,000 times higher concentrations caused a slightly lower response, but this difference was not significant (Table 1). The addition of monoterpenes at the strongest dosage of 10^{-5} g/μL had no effect on attraction in either sex to the field dispensers of G1 and cV (Table 1).

In a second series of bioassays, a dosage of 10^{-9} g/μL G1+cV that alone attracted 68.3% females was combined

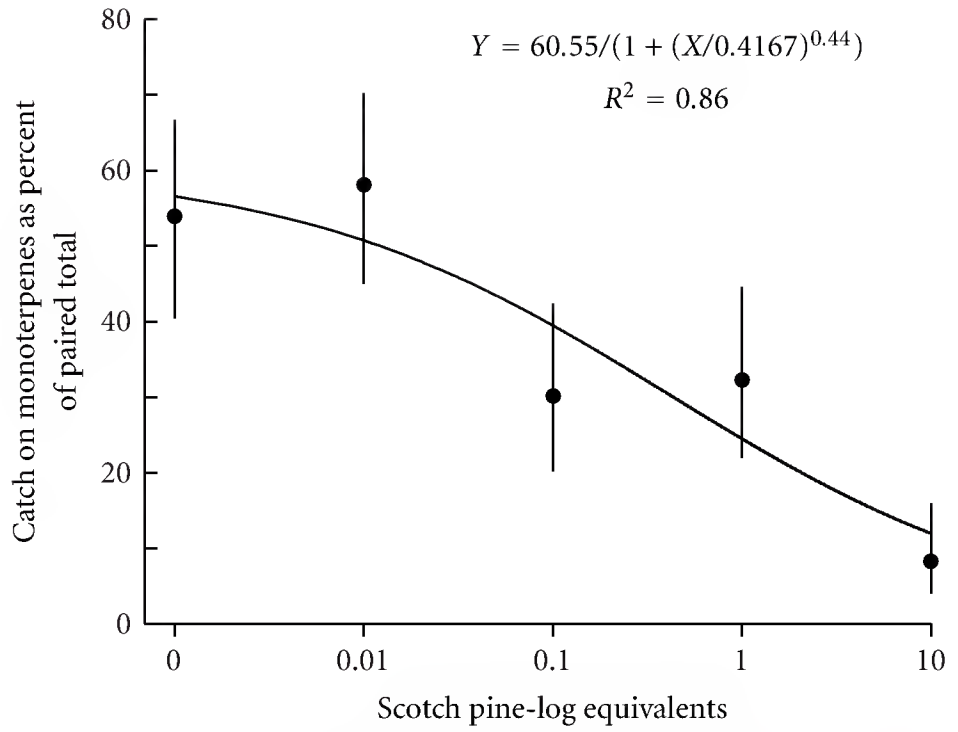

FIGURE 3: Inhibition of *P. bidentatus* response by increasing release rates of a mixture of Scotch pine monoterpenes ((−)-α-pinene, (+)-α-pinene, (+)-3-carene, and terpinolene) each released in proportion to release of 1.0 Scotch pine log-equivalent (10 μg/min) in hexane with the piezoelectric sprayer from one of a pair of slowly-rotated, pheromone (*cis*-verbenol and grandisol)-baited traps (Veberöd, Sweden, 19 May 1999; data from [9]). Error bars represent 95% binomial confidence limits for the proportion trapped in the monoterpene-releasing trap relative to the total catches by the pair.

with the strongest dosage tested of monoterpenes (10^{-5} g/μL or neat monoterpenes), but their response was not reduced (Table 2). Walking males responded similarly as females to the aggregation pheromone components and there was no evidence of any inhibition by host tree monoterpenes released neat from the 5-μL pipette (Table 2). The releases of neat monoterpenes in the open-arena olfactometer for α-pinene, 3-carene, and terpinolene were 28, 9.4, and 4.8 μg/min, respectively.

A third series of bioassays a year later was performed in which the release rate of the aggregation pheromone components was increased over several orders of magnitude from 5×10^{-10} to 5×10^{-7} g/μL, which resulted in an increasing response of walking females and males to the source (Table 3). But again, a strong release of 10^{-5} g/μL of monoterpenes, either (−)-β-pinene (a major monoterpene of non-host Norway spruce but not of host pine) or (−)-α-pinene (a major monoterpene in both Scotch pine and Norway spruce), did not decrease response to an optimal dosage (5×10^{-7} g/μL) of the aggregation pheromone components (Table 3).

4. Discussion

The results of selected tests from the three previous studies [7–9] showed strong to moderate inhibition of the flight orientation of both sexes of *P. bidentatus* to synthetic aggregation pheromone components in the field (Figure 1). Norway spruce bark was highly inhibitory as well as spruce needles, but even the host tree Scotch pine bark or needles were strongly inhibitory (Figure 1). Mixtures of monoterpenes as well as five individual host monoterpenes clearly cause flying *P. bidentatus* of both sexes to avoid landing on traps releasing aggregation pheromone when compared to traps releasing the same rate of pheromone without inhibitory volatiles (Figures 1 and 2). The current field tests (Figure 2), show that *P. bidentatus* of both sexes avoid landing in areas with aggregation pheromone if the monoterpenes myrcene, (−)-β-pinene, terpinolene, (−)-α-pinene, and (+)-α-pinene are also released. Only females were significantly inhibited by (+)-3-carene, while neither sex appeared to avoid either enantiomer of limonene when orienting to aggregation pheromone (Figure 2).

In the laboratory bioassay with walking beetles, however, several monoterpenes released with aggregation pheromone components had no apparent effect on attraction (Tables 1–3). The large range of monoterpene release rates over three orders of magnitude in the laboratory bioassay are estimated to correspond to natural release rates from small wounds to broken limbs of conifers [14, 15], and were similar to the rates that elicited behavioral activity (attraction) from *Tomicus piniperda* (L.) and *T. minor* (Hart.) in the same laboratory olfactometer [14–16]. The highest concentration of ether-diluted monoterpenes tested in the laboratory olfactometer (10^{-5} g/μL) had a maximum estimated release rate of 1300 μg/h, which is comparable to the release rate in field trials (i.e., 1000 to 1400 μg/h) that caused inhibition of attraction [7]. The actual release rates of monoterpenes dissolved in diethyl ether from the 5-μL capillary, however, were probably different due to differing vapor pressures of the monoterpenes and diethyl ether [41]. However, the highest release rates of neat monoterpenes in the laboratory walking bioassay were 1680 μg/h for each enantiomer of α-pinene, 565 μg/h for 3-carene, and 289 μg/h for terpinolene, and these rates were estimated to be equivalent to the release of the major monoterpenes from three freshly cut logs of Scotch pine (30 cm × 13 cm diameter) [9, 14, 15]. In contrast to walking beetles in the olfactometer, attraction by flying *P. bidentatus* to aggregation pheromone in the field was significantly reduced by these same monoterpenes at 0.1 log-equivalent (60 μg/h) or 1 log-equivalent (600 μg/h) dispensed from a piezoelectric sprayer (Figure 3) [9]. Thus, release rates of monoterpenes that failed to inhibit attraction to pheromone by walking beetles in the laboratory were equivalent [7] or higher [9] than rates that inhibited attraction of flying beetles in the field.

Natural selection should favor conifer-infesting bark beetles that find their host tree by keying on pheromones and/or host volatiles of which the monoterpenes are the most abundant. Some bark beetles such as *T. piniperda* and to a lesser extent *T. minor* are strongly attracted to monoterpene vapors emanating from resinous wounds incurred when the trees fall during winter and spring storms [14–16]. Monoterpenes are also weakly attractive to secondary bark beetles that follow the tree-killing beetles after the tree succumbs [17]. (−)-α-Pinene weakly attracted *Ips grandicollis* (Eichhoff) and *Dendroctonus valens* LeConte but not *I. avulsus* (Eichhoff) or *I. calligraphus* (Germar) [23, 25]. Bark beetles that kill their host tree in a mass attack in the genera *Ips* and

Dendroctonus are weakly or not attracted by monoterpenes alone ([23, 25, 44], Byers unpublished) although in some cases specific monoterpenes can enhance responses to aggregation pheromone, as occurs in *D. brevicomis* LeConte, *D. pseudotsugae* Hopkins, *D. valens*, *D. frontalis* Zimmermann, and *I. grandicollis* [20–25]. This also seems to be the case for tree-killing *P. chalcographus* that attack Norway spruce; both sexes were more attracted to aggregation pheromone components when either enantiomer of α-pinene, $(-)$-β-pinene, or camphene was coreleased [18].

The aggressive bark beetles generally are not attracted to susceptible hosts by means of monoterpenes, but are believed to select hosts by a process of randomly landing on trees and determining their resistance level by boring through the outer bark [45, 46]. If the tree produces enough resin, then the beetle must leave or be killed. If the beetle succeeds in feeding due to low host resistance, then aggregation pheromone is released that attracts many more individuals to overcome the tree. Thus, the vast majority of individuals in the tree-killing bark beetle species find their host tree by orienting to aggregation pheromone [46, 47]. *P. bidentatus* appears to be much less aggressive, preferring to colonize only broken or weakened limbs of pine in which resin production as a defense is greatly reduced or nonexistent [1–4]. As the tree becomes colonized and begins to degrade, higher rates of verbenone and ethanol are released that reduce attraction of many of the bark beetle species to pheromone or host monoterpenes [11, 15, 17, 33, 48–50]. In *P. bidentatus*, however, ethanol reduced, while verbenone did not reduce, response to aggregation pheromone in tests with trap pair slow-rotation [7].

The open-arena olfactometer used here is a bioassay that has been tested on several bark beetle species, and individuals of both sexes commonly behave in a way that is consistent with trap captures in the field. For example, walking *I. paraconfusus* are increasingly attracted to higher doses of aggregation pheromone components in the laboratory [39, 51], but at the higher release rates the males are increasingly less attracted, just as in the field [51]. In *D. brevicomis*, walking females and males are increasingly attracted to their aggregation pheromone components in the olfactometer, and release of male-produced verbenone causes both sexes to avoid the aggregation pheromone source; the same behaviors that occur when flying beetles respond to baited traps in the field [40, 41]. Both sexes of *D. brevicomis* produce *trans*-verbenol during feeding in ponderosa pine [52], and only females are inhibited by higher concentrations of $(-)$-*trans*-verbenol either when walking in the olfactometer or when entering holes in carton traps in the field [42]. Another study with the olfactometer has shown that walking *T. piniperda* are attracted to host monoterpenes as well as similarly attracted when flying to host logs or monoterpenes in sticky traps [14]. Walking *P. chalcographus* are increasingly attracted in the laboratory olfactometer to increasing release of their pheromone components, and subtraction of the monoterpene fraction from an odor collection of a male-infested log caused a moderate decrease in attraction [19]. This is in agreement with a field study showing monoterpenes increasing flight attraction and proportionally greater entry of walking *P. chalcographus* through small holes into traps releasing aggregation pheromone components [18].

Thus, in all cases except the present study, bark beetle behavior in the open-arena olfactometer has been in general agreement with the behavior observed in the field with traps baited with semiochemicals. If there were different bioassay operators, this might explain the discrepancy between flight and walking behaviors in *P. bidentatus*; however, the same person (Byers) performed all laboratory bioassays discussed above [14, 18, 19, 39–42, 51, 52]. Rather, the differences appear to be explained by the ecology of *P. bidentatus* that appears less aggressive and colonizes unhealthy branches, in contrast to the pest bark beetles mentioned above that often kill trees. Six possible combinations of behavior could have evolved in *P. bidentatus* when responding to aggregation pheromone in regard to whether to respond, ignore, or avoid monoterpene odors while flying or walking. The best adaptation for a less aggressive bark beetle such as *P. bidentatus* that prefers to colonize weakened and diseased branches or smaller unhealthy trees would be to avoid monoterpenes when flying (so as to not land in resin and more efficiently select suitable hosts from among resistant hosts and nonhosts) but not when walking (so as to enter holes made by mates or excavate entrance holes where concentrations of monoterpenes are higher). Other more aggressive bark beetles may not avoid monoterpenes in combination with aggregation pheromone while flying because these species are suited to tolerate resin when overcoming the tree's resistance [11].

The monoterpenes were not tested alone in the rotating traps in the field or in the laboratory bioassay so it is not known if they could be attractive to *P. bidentatus* at some concentration. However, Byers [17] tested monoterpenes (enantiomers of α-pinene, 3-carene, and terpinolene, each at 104 μg/h to 583 μg/h) or a combination of ethanol and monoterpenes in the field during the spring in the same location as the present study; neither *P. bidentatus* nor any other *Pityogenes* species were caught even though five other bark beetle species were attracted.

It is proposed that pheromone-producing bark beetles that are not attracted to monoterpenes or other host-associated odors may initially land on trees at random in response to the visual silhouette of the trunk [45, 51]. Once a beetle bores into a suitable tree and is able to produce aggregation pheromone, a mass colonization ensues. The presence of aggregation pheromone indicates an ongoing colonization by *P. bidentatus* and thus is a cue to the likely presence of a weakened host and a valuable resource to be exploited. Conifers usually produce resinous wounds in response to mechanical damage from storms or other causes, and the exposed resin poses a hazard to bark beetles orienting to and attempting to colonize these trees. The visual acuity of bark beetles, with about 200 facets per eye [11], probably does not allow beetles to differentiate resin globules and resinous patches on a tree trunk or branch before they land. *T. piniperda*, *I. typographus* L., and *P. chalcographus* beetles walking on the bark of wounded conifers in Sweden that happened to contact resin globules were seen to back away and turn to find a path free of resin (personal observations).

It is probable that *P. bidentatus* similarly avoid resin while walking. It is also expected that fitness of *P. bidentatus* would be enhanced when responding to aggregation pheromone if they could avoid both potentially fatal landings in resinous patches and bark unsuitable for colonization due to a tree's vigorous resin defenses. In addition, flying beetles would save time and energy by avoiding monoterpenes of fresh host trees as well as nonhost trees in the vicinity of a suitable host. Healthy trees capable of exuding resin from wounds probably would be unsuitable hosts for *P. bidentatus*, since they typically attack diseased and dying branches possessing compromised resin defenses. After landing, the beetles may seek out the source of aggregation pheromone while relying on their ability to back away from any sticky resin they encounter while walking.

It is likely that no species of conifer-infesting bark beetle would be repelled by monoterpenes while walking on the bark surface and orienting to pheromone, since they must encounter high concentrations of monoterpenes either when they enter the gallery of a mate or initiate a new gallery. To my knowledge, no tests of bark beetles walking in laboratory olfactometers and orienting to aggregation pheromone have shown monoterpenes to be repellent. *P. bidentatus* is not the only bark beetle that is inhibited by host volatiles in flight since *I. avulsus* response to aggregation components was inhibited by high releases of loblolly pine turpentine (undefined mixture of monoterpenes at 150,000 μg/h) [25] and *I. pini* (Say) flight response to its aggregation components was inhibited by racemic α-pinene at high rates (23,000 μg/h) [24]. It is not known how *I. pini* or *I. avulsus* respond to monoterpenes and aggregation pheromone when walking.

Further work with *P. bidentatus* is needed to understand the conditions and benefits of ignoring monoterpene odors while walking but avoiding these odors when flying. It appears remarkable that this tiny insect is able to exhibit two types of behavior in regard to aggregation pheromone and monoterpenes. The first behavior of avoiding monoterpenes when flying seems adaptive in that a beetle averts plunging into sticky resin that could entrap them and saves time/energy during searches for suitable hosts. The second behavior of ignoring monoterpene vapors when walking also seems adaptive to find colonization areas and entrance holes of mates on the bark, and escaping any encountered resin by backing away. The possibility of dichotomy in behaviors when flying and walking needs to be investigated in other species of bark beetles to understand the adaptive benefits.

Acknowledgments

This study was supported by grants from the Swedish Agricultural and Forestry Research Council (SJFR) to the Swedish University of Agricultural Sciences, Alnarp, Sweden.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 269473, 9 pages
doi:10.1155/2012/269473

Review Article

Host-Symbiont Interactions for Potentially Managing Heteropteran Pests

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Received 27 February 2012; Accepted 27 April 2012

Academic Editor: Jeffrey R. Aldrich

Insects in the suborder Heteroptera, the so-called true bugs, include over 40,000 species worldwide. This insect group includes many important agricultural pests and disease vectors, which often have bacterial symbionts associated with them. Some symbionts have coevolved with their hosts to the extent that host fitness is compromised with the removal or alteration of their symbiont. The first bug/microbial interactions were discovered over 50 years ago. Only recently, mainly due to advances in molecular techniques, has the nature of these associations become clearer. Some researchers have pursued the genetic modification (paratransgenesis) of symbionts for disease control or pest management. With the increasing interest and understanding of the bug/symbiont associations and their ecological and physiological features, it will only be a matter of time before pest/vector control programs utilize this information and technique. This paper will focus on recent discoveries of the major symbiotic systems in Heteroptera, highlighting how the understanding of the evolutionary and biological aspects of these relationships may lead to the development of alternative techniques for efficient heteropteran pest control and suppression of diseases vectored by Heteroptera.

1. Introduction

Insects are the most cosmopolitan, polyphagous, and varied living organisms on Earth, and many species are involved in some kind of symbiotic association with microorganisms, mainly bacteria [1, 2]. Some insects associated with bacteria are also vectors of disease or are important crop pests which increase the relevance of these symbiotic interactions. The widespread distribution of insects may, in fact, be related to bacterial associations that allow host insects to exploit different nutritional sources, such as lignocellulose by termites, or to obtain essential nutrients from their symbionts, as in *Buchnera aphidicola*-aphid symbiosis [3, 4].

According to Moran [2], symbiosis is a "close relationship between two or more individuals." In insects, there are two major categories of symbiotic associations: obligatory and facultative [5]. Obligatory symbionts (also called primary symbionts) are nutritionally required for the survival of their insect hosts and usually inhabit specialized host cells. On the other hand, secondary (also known as facultative) symbionts can be beneficial or cause incidental or deleterious infections [1]. The obligatory symbiont, *B. aphidicola*, for example, has never been cultured outside its host and is present intracellularly within specialized cells termed bacteriocytes [1, 5, 6]. Transovarial transmission is often the mode of symbiont transfer from one generation to another, which is a bottleneck that shapes the genome characteristics of the symbiont [7]. Phylogenetic analyses for certain insect families have shown that insects and primary endosymbionts have coevolved for millions of years after a single initial infection [5]. In contrast, secondary symbionts are nonessential to their hosts, may be free living, may not have specialized tissue localization, and occur extracellularly [8]. These secondary symbionts may provide benefits to their hosts such as tolerance to heat stress, compensation for loss of primary symbionts, and resistance to parasites and pathogens [9–13]. Conversely, facultative symbionts can negatively impact the growth, reproduction, and longevity

of their hosts [14]. The evolutionary history of secondary endosymbionts often shows no coevolution with their hosts, suggesting multiple infections and/or horizontal transmission [5, 15]. For example, the secondary endosymbiont of tsetse flies, *Sodalis glossinidius*, can be cultured *in vitro* and apparently has not coevolved with the insect hosts [16]. *Sodalis glossinidius* is closely related to bacterial pathogens of insects suggesting, in this case, that the symbiont evolved from an insect pathogen [16].

In Heteroptera, many species adversely affect humans and their environment by causing direct damage to the crops, or acting as vectors of disease to crops, domestic animals, and humans [17] (however, there are also many agriculturally beneficial predatory heteropterans). For over 50 years, it has been known that insects of the suborder Heteroptera (order Hemiptera) harbor symbiotic microorganisms; however, the significance of the relationship and their role in the host's ecology and evolution are only now being unraveled [1, 9]. Symbiotic bacterial associations occur in all three hemipteran suborders: Sternorrhyncha (e.g., aphids, mealybugs, whiteflies, psyllids, etc.), Auchenorrhyncha (e.g., spittlebugs, planthoppers, leafhoppers, treehoppers, etc.), and Heteroptera (true bugs; Figure 1) [5, 18–21]. Experimental procedures for the phylogenetic tree analysis of Figure 1, the almost complete 16S rRNA gene sequences (1,300 nucleotides) were obtained from GenBank database and were aligned manually using MEGA version 5 software [22]. Phylogenetic trees were inferred by using the maximum-likelihood [23], maximum-parsimony [24], and neighbor-joining [25] tree-making algorithms drawn from the MEGA 5 and PHYML packages [22, 26]; an evolutionary distance matrix for the neighbor-joining algorithm was generated using the Jukes and Cantor [27] model. The topologies of the evolutionary trees were evaluated by a bootstrap analysis [28] of the neighbor-joining method based upon a 1,000 replicates using the MEGA 5 software.

Heteropteran symbionts are found in the gut lumen, as in Reduviidae, or in gastric caecae as in Acanthosomatidae, Alydidae, Coreidae, Parastrachiidae, Pentatomidae, Pyrrochoridae, Plataspidae, and Scutelleridae. As opposed to symbionts of Sternorrhyncha and Auchenorrhyncha, the symbionts of Heteroptera are not passed to the next generation from mother to offspring in a transovarial manner. The posthatch transmission mechanisms may involve egg surface contamination (= smearing on egg surface), coprophagy (= proctophagy: feeding on excrement), capsule transmission, or acquisition from the environment "*de novo*" every generation after the nymphs hatch [1, 18, 19, 21, 29–39]. This curious mode of transmission is challenging to both the host and symbiont. In particular for the symbiont, external transmission may require high genome stability to prevent the loss of genes required for living outside the host in a variable environment, and the challenge for the host bugs is to reinoculate themselves each generation. These challenges may be responsible for the multiple acquisition of the symbiont or low cospeciation observed in Heteroptera [21, 29, 40].

Not all heteropteran microbial associations are beneficial to the host; at times the bacteria may be pathogenic and reduce fitness (i.e., reproduction, mortality, and longevity) [41]. The beneficial and pathogenic aspects of symbiosis have been studied in the past, but now novel molecular approaches are being applied to these systems. With the advent of molecular approaches, it is increasingly clear that manipulation of symbiotic interactions can contribute to the development of new strategies for pest control, including the use of modified symbionts to control insects (paratrangenesis) [30, 42], replacement of native symbionts with genetically modified symbionts via genetic drive [43, 44], and a technique called "incompatible insect" [45]. Additionally, Broderick et al. [46] showed that *Bacillus thuringiensis*, widely applied in biocontrol projects, only kills the lepidopteran larvae if the insects harbor a gut-associated microbial community, highlighting the importance of gut-associated bacteria to pest control.

2. Reduviidae

The vectors of the Chagas disease pathogen are bloodsucking Reduviidae in the subfamily Triatominae. For example, *Rhodnius prolixus* (Stal) is a blood-sucking triatomine that is a common vector of Chagas disease, the incurable illness damaging the heart and nervous system that afflicts millions of people in Central and South America [42]. This insect acquires its bacterial symbiont, *Rhodococcus rhodnii,* soon after the first instar bug hatches. In triatomines, generally, the aposymbiotic (without symbiont) first instar nymphs hatch and probe for their bacterial symbionts that are acquired orally through "contamination" by feces on or nearby egg masses [1, 30, 47]. This insect-symbiont association has been exploited by paratrangenesis, in which the symbiont has been genetically transformed to negatively interfere with the survivorship of the Chagas disease agent, *Trypanosoma cruzi* [48]. Genetically modified *R. rhodnii* symbionts expressing a selectable gene product were stably maintained in *R. prolixus* without deleterious effects on host survival and fitness, thereby substantiating the paratransgenic approach.

According to Hurwitz et al. [49] the paratransgenic strategy has been used with other vector-borne disease systems such as sandfly-mediated leishmaniasis and sharpshooter-mediated Pierce's disease [50–52]. Additionally, the authors highlight the main strategies for the success of the paratrangenesis strategy.

(1) In order to perform genetic manipulation, it is necessary that the symbiont can be cultured.

(2) The symbiont must be identified within a certain disease-transmitting vector.

(3) After the genetic manipulation, insect and symbiont fitness should not be negatively impacted.

(4) The transgene product expressed must interfere with pathogen development in the vector, but should not affect the fitness of the vector.

(5) The technique used to spread the genetically modified symbiont/commensal to naturally occurring vector populations should minimize the spread of

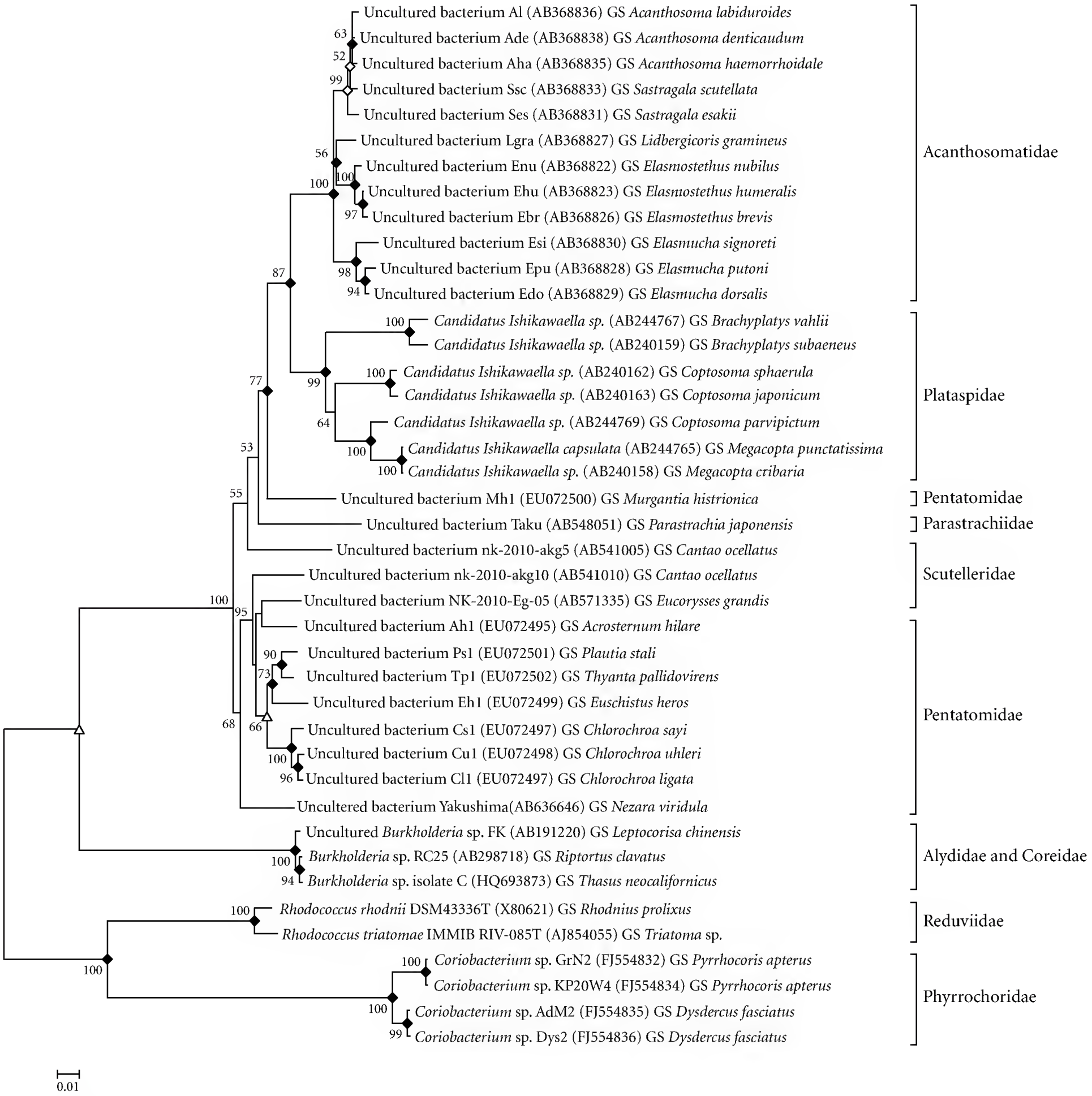


Figure 1: Neighbor-joining tree based on nearly complete 16S rRNA gene sequences (1,300 bp) showing phylogenetic relationships between gut symbionts (GS) of heteropteran species. Insect families are indicated after brackets. Black diamonds indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making algorithms; white triangle and diamonds stand for branches that were recovered by the maximum-likelihood or by the maximum-parsimony tree-making algorithms, respectively. Numbers at the nodes are percentage bootstrap values based on a neighbor-joining analysis of 1,000 resampled datasets; only values above 50% are given. Bar 0.01 substitutions per nucleotide position.

the transgene to other organisms in the vector's environment, which include both the nontarget microbes inside the host/vector and other organisms that live in the same ecological niche.

3. Acanthosomatidae

This family, a member of the stink bug or shield bug Pentatomoidea superfamily, is characterized by social behavior, which features the maternal instinct to guard eggs and nymphs against possible predators [53]. The genetic and evolutionary characterization of the caeca-associated symbionts in 14 different species of Acanthosomatidae, representing a total of five genera (*Elasmostethus*, *Lindbergicoris*, *Elasmucha*, *Sastragala*, and *Acanthosoma*), has been elucidated [33]. Acanthosomatid bugs harbor extracellular symbionts of a specific clade of Gammaproteobacteria in midgut crypts.

In Acanthosomatidae, both host and symbiont have co-speciated together, and the vertical symbiont transmission is via egg surface contamination [33].

4. Alydidae and Coreidae

Alydidae (called broad-headed bugs) and Coreidae (leaf-footed bugs) are relatively small, principally herbivorous families. Alydid and coreid bugs feed mostly on seeds, less often on the phloem, of various plants; alydids also occasionally exhibit coprophagy and carrion feeding [17]. The broad-headed bugs, *Riptortus clavatus* (Thunberg), *Leptocorisa chinensis* (Dallas), and the giant mesquite bug *Thasus neocalifornicus* Brailovsky and Barrera, harbor symbionts of the *Betaproteobacteria* type in the genus *Burkholderia*. These insects acquire symbionts from the soil (i.e., horizontally) in each generation and harbor the bacteria in the lumen of crypts situated along the midgut [15, 54, 55]. Inoculation of aposymbiotic nymphs with cultured symbiotic microorganisms and comparison with aposymbiotic adults reared under sterile conditions suggest that the absence of the symbiont decreases host fitness [15, 54]. Moreover, phylogenetic analysis shows that the *Burkholderia*-like sequences from the digestive tract of *T. neocalifornicus* are closely related to those found in *L. chinensis* and *R. clavatus*, data acquired by amplifying the 1.5-kb segment of the eubacterial 16S rRNA gene [15].

5. Parastrachiidae

In Parastrachiidae (another pentatomoid family), *Parastrachia japonensis* Scott is monophagous, feeding of drupes of the deciduous tree *Shoepfia jasminodora* (Santalales: Olacaceae) [56]. The parastrachiid mother provides food for her nymphs, and the ensuing adults enter into diapause for 9 months, surviving only on water. Molecular phylogenetic analyses of *P. japonensis* symbionts revealed that they constitute a distinct phyletic line in the Gammaproteobacteria 16S rRNA gene subclade. This parastrachiid symbiont has no close relatives, but is allied with gut symbionts of acanthosomatid and plataspid bugs, as well as with endocellular symbionts of sharpshooters, tsetse flies, and aphids [36]. According to Kashima et al. [57], this symbiont might be involved in the uric acid recycling system due to the increased mortality of the adults when they were treated with antibiotic during the nonfeeding period.

6. Pentatomidae

Within Heteroptera, the Pentatomidae (the true "stink bugs") is one of the largest families with over 4000 species [17]. Many pentatomid insects are polyphagous, feeding on a diverse range of plants. Stink bugs are economically important pests throughout the world on a multitude of crops, including soybeans, rice, pecan, cocoa, and macadamia nuts to name a few [17, 58]. They can cause direct and indirect damage, as can other heteropteran, by feeding on plant tissue with needle-like stylets, injecting digestive enzymes into plant tissue, or providing free access

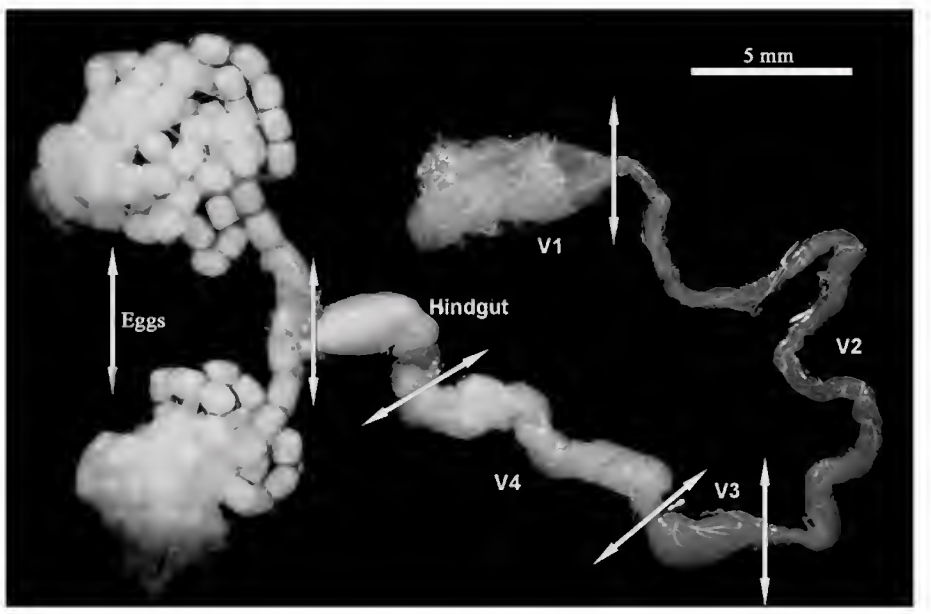
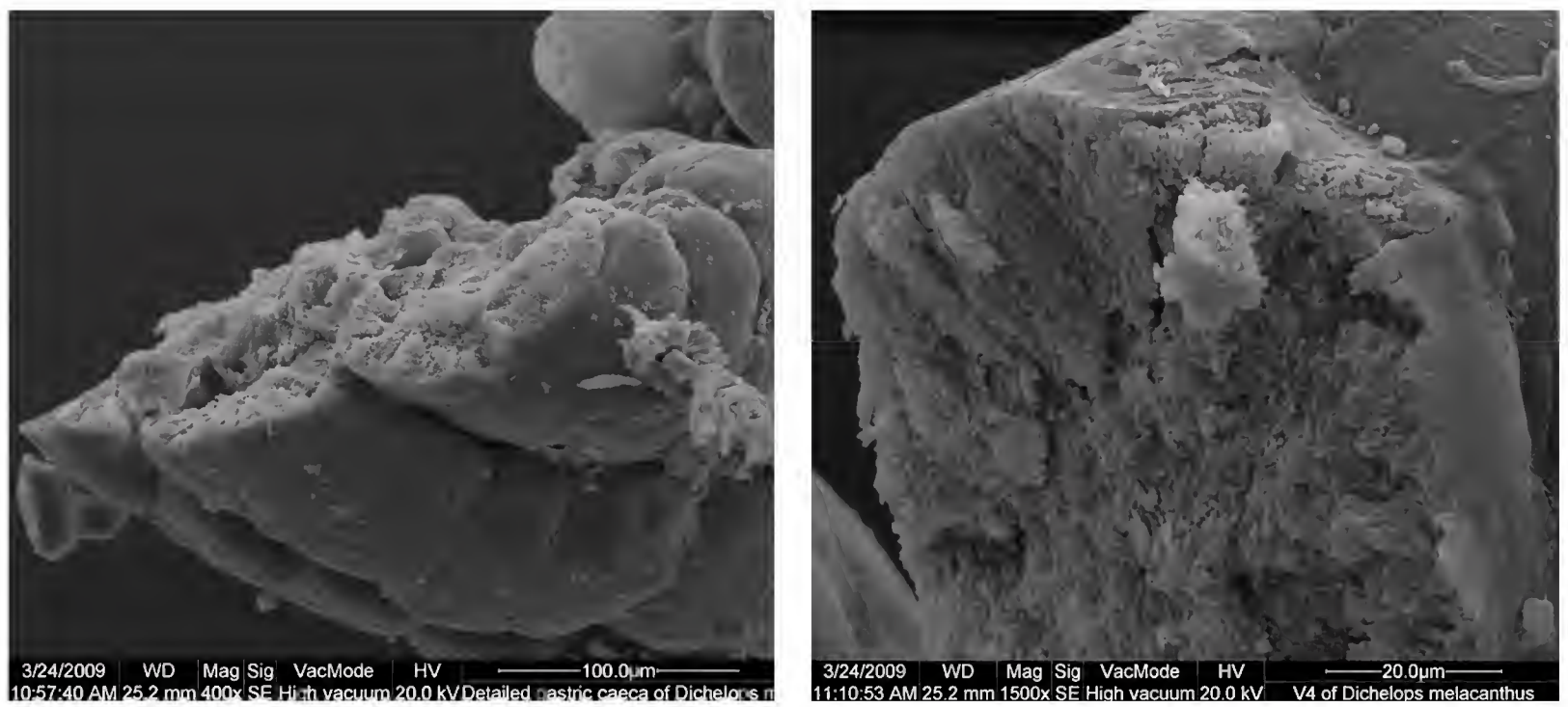


Figure 2: Detailed figure of the midgut of *Nezara viridula* divided into four ventricles. V4 is the gastric caeca, where the symbionts are located [21].

to microbial infection [17, 59]. The economic importance of stink bugs is magnified as they are usually difficult to control [60]. Recently, it was shown that the stink bugs, *Acrosternum hilare* (Say), *Chlorochroa ligata* (Say), *Chlorochroa sayi* (Stal), *Chlorochroa uhleri* (Stal), *Dichelops melacanthus* (Dallas), *Edessa meditabunda* (F.), *Euschistus heros* (Fabricius), *Loxa deducta* Walker, *Murgantia histrionica* (Hahn), *Nezara viridula* (L.), *Pellaea stictica* (Dallas, 1851), *Piezodorus guildinii* (Westwood), *Plautia stali* Scott, *Thyanta pallidovirens* (Stal), and, *Thyanta perditor* (F.) are associated with plant pathogens (*Pantoea* spp.) contained in the gastric caecal region (ventricula 4; Figures 2 and 3) of their midguts [21, 34, 35, 40, 61, Prado, S.S. unpublished data]. In general, stink bug symbionts are polyphyletic, although some degree of monophyly has also been observed suggesting that the symbionts were probably acquired and occasionally replaced by other bacteria over evolutionary time [34, 35]. Smearing of symbionts on the egg surface by ovipositing females and subsequent acquisition of the symbiont by aposymbiotic first instar nymphs appears to be the mechanism of vertical transmission.

The cosmopolitan pentatomid, *Nezara viridula*, is both generally and obligatorily associated with a gut symbiont; however, it seems that the type and duration of the association is somewhat different between populations based on the geographical region where the insect is found [20, 21, 39]. At 30°C, *N. viridula*'s symbiont maintenance is affected and insect development is accelerated [40]. Insects free of the symbionts reared at 20°C had longer mean nymphal developmental time, and females never laid eggs [21, 40].

In addition, *P. stali*, when deprived of its gut-associated symbiont, has a slower developmental time than individuals with the symbiont [29]. For *A. hilare*, the elimination of the symbiont by surface sterilization of egg masses negatively impacted development and reproduction [35]. Conversely, the absence of *M. histrionica*'s gut symbiont seems to have no effect on the development of the insect host; however, when both species (*A. hilare* and *M. histrionica*) were reared at 30°C, each lost their respective symbiont [61].



(a) (b)

(c) (d)

FIGURE 3: Detailed figure of scan electron microscopy (SEM) of the gastric caeca of *Dichelops melacanthus* in (a), detail of the amount of bacteria inside the gastric caeca in (b), and bacteria detail in (c) and (d).

The impact of surface sterilization on the maintenance of the symbionts and on the development of *E. heros*, *D. melacanthus,* and *P. stictica* is being evaluated by Prado et al. (unpublished). Data thus far has shown that a decrease in host fitness was associated with, and probably mediated by, symbiont loss at 30°C. This suggests that, not only egg mass sterilization, but also higher temperature may affect population performance of the insects directly or indirectly through mediated effects on their mutualists [35, 61]. The role of these vertically transmitted pentatomid gut symbionts, therefore, appears to vary for different bug host species. For instance, the cabbage stink bugs, *Eurydema rugosa* Motschulsky and *Eurydema dominulus* (Scopoli), also have symbionts associated with their gastric caecae; absence of the caeca-associated symbiont due to surface sterilization of egg masses caused retarded growth, reduced body weight, and abnormal body color [62].

Recently, using genus-specific primers and appropriate PCR conditions, Zucchi et al. [63] characterized an earlier unnoticed community of actinobacteria inhabiting the gastric caeca of several pentatomid species (Table 1). Although only a few insects species have been reported associated with *Actinobacteria* [64], the best-known case involving nutrient provision (see Section 2), studies on pentatomids have pointed to an alternative beneficial association in which the actinobacteria produce an antibiotic barrier against pathogens [65, 66]. The role of these actinobacteria in the midgut of the stink bug is still unknown, but Zucchi et al. [63] speculate that the actinobacteria byproducts may regulate the gastric caecal bacterial community.

7. Phyrrochoridae

There is at least 262 pyrrochorid species distributed in 29 genera, with *Dysdercus* being the most important and largest genus [17]. These bugs are called cotton stainers because their excreta, plus the disease organisms they admit, stain cotton fiber. An actinobacterium, *Coriobacterium glomerans,* has been described as the extracellular gut-associated

Table 1: Actinobacteria diversity inhabiting the midgut of Pentatomidae.

Family	Genus	Host
Actinomycetaceae	*Actinomyces* spp.	*Thyanta perditor*
Brevibacteriaceae	*Brevibacterium* spp.	*Dichelops melacanthus*
Corynebacteriaceae	*Corynebacterium* spp.	*Dichelops melacanthus* *Edessa meditabunda* *Thyanta perditor*
Dietziaceae	*Dietizia* spp.	*Dichelops melacanthus* *Loxa deducta* *Pellea stictica*
Intransporangiaceae	*Ornithinimicrobium* spp.	*Loxa deducta* *Pellea stictica*
Kineosporiaceae	*Kineococcus* spp.	*Dichelops melacanthus*
Microbacteriaceae	*Microbacterium* spp.	*Edessa meditabunda*
Micrococcaceae	*Arthrobacter* spp. *Citrococcus* spp.	*Edessa meditabunda* *Dichelops melacanthus* *Edessa meditabunda*
Mycobacteriaceae	*Mycobacterium* spp.	*Loxa deducta* *Pellea stictica* *Piezodorus guildinii* *Thyanta perditor*
Propionibacteriaceae	*Propionibacterium* spp.	*Dichelops melacanthus* *Piezodorus guildinii*
Streptomycetaceae	*Streptomyces* spp.	*Nezara viridula*

Source: modified from Zucchi et al. [63].

symbiont in *Pyrrhocoris apterus* (L.) and *Dysdercus fasciatus* Sign. [32, 67, 68]. Recently, Kaltenpoth et al. [32] showed that the bacterial symbionts are located mainly in the third part of the midgut (V 3), with cells found connected to the epithelium and swimming freely in the gut. The symbionts are primarily transmitted vertically by egg smearing, but horizontal transmission also occurs [32]. The bacterial cells can form long chains in the gut of the insects, where they are assumed to aid in digestion [67].

8. Plataspidae

Insects of this family are almost entirely from the tropical old world most species are Oriental and; this is one of a few groups that feed most of the time on legumes [17]. The Japanese common plataspid, *Megacopta punctatissima* (Montandon), harbors the bacterial symbiont *Candidatus* Ishikawaella capsulata in its gastric caeca [19]. Fukatsu and Hosokawa [18] showed that after hatching, the aposymbiotic first instar nymphs immediately probe small brownish capsules attached to the eggs masses laid by the females in order to acquire their symbiont [18, 19, 41]. In addition, Hosokawa et al. [19] used phylogenetic reconstruction to show that both insect and symbiont have undergone cospeciation and when deprived of its symbiont, *M. punctatissima*'s growth and survival are negatively influenced [18, 19, 41]. The plataspid bug, *Megacopta cribraria* (Fabricius), also has an obligatory relationship with its primary endosymbiont, which is similar to that first described for *M. punctatissima* [69]. Recently, Hosokawa et al. [41] showed by experimentally exchanging the obligatory gut-associated symbiont between *M. punctatissima* and *M. cribraria*, that the success of the important pest species on legumes (*M. punctatissima*) was negatively impacted due to high nymphal mortality before or upon hatching. Conversely, *M. cribraria*, which is considered a nonpest species of the legume crop, when carrying *M. punctatissima*'s obligatory symbiont, exhibited the attributes of the naturally pestiferous species. These exciting findings raise new hypotheses on the evolutionary origin of an insect pest, which may lead to the development of alternative methods to control and manage species considered pests [41].

9. Scutelleridae

Within the Pentatomoidea, this family (commonly called shield bugs) is most closely related to Pentatomidae. All scutellerids are phytophagous, but only a few have been reported as pests [17]. The giant jewel shield bugs, *Cantao ocellatus* (Thunberg) and *Eucorysses grandis* (Thunberg), possess a gammaproteobacterial primary gut symbiont and a *Sodalis*-allied secondary symbiont [37, 38]. The specific

bacterium from *E. grandis* was consistently identified in insects from five different geographic regions and was detected in 100% of the insects surveyed from three host populations. Molecular phylogenetic analysis clearly showed that the primary gut-associated symbiont of *E. grandis* constitutes a distinct lineage in the *Gammaproteobacteria*, and is closely related neither to the gut symbiont associated with *C. ocellatus*, nor to gut symbionts of other stink bugs, suggesting that scutellerid symbionts have multiple evolutionary origins [38].

10. Concluding Remarks

In this paper, we reviewed major trends in symbiotic association for diverse members of the Heteroptera. These interactions have been primarily studied for their ecological interest regarding insect development. In the past decade, it has become increasingly clear that exploiting these relationships may be a fruitful alternative type of biological control; paratrangenesis [42] and specific elimination of the essential bacteria of the pest or vector insect [70] demonstrated promising results.

Endosymbiotic bacteria of insects have received considerable attention in the past few decades. Many studies focused on the intimate associations of intracellular symbionts, their hosts and the degree of mutual interdependence of these symbioses. Most of these studies focused on obligatory symbionts that are difficult or so far impossible to cultivate. Successful attempts at pest control or disease management, such as those demonstrated by the paratrangenesis of the endosymbiont actinobacteria from triatomine species, should become more common once efforts to identify other bacterial symbionts for other heteropteran hosts are more successful, particularly with secondary symbionts. Furthermore, recent studies have shown that extracellular gut symbionts of insects can engage in symbiotic interactions of similar intimacy and specificity with their hosts, and may exhibit similar evolutionary and genomic consequences of the symbiotic lifestyle [19].

There may also be negative impacts on the symbiont-host relationship caused by global warming changes and, consequently, interference in insect survivorship and ecology from elevated global temperatures, encouraging more research on these associations [9, 12, 14, 15, 71].

At the time of writing, only a few examples of insect-microorganism associations are effectively being explored for control of pests or human diseases. This is still an open area of research with great potential for control of insect pests and vectors of disease, as the cases mentioned earlier using the paratransgenic strategy in the systems of sandfly/leishmaniasis and sharpshooter/Pierce's disease represent [50–52]. In fact, it is only recently that considerable information has been gathered to permit the design of alternative methods of control. Studies on different bacterial groups, such as actinobacteria, reminds us how intricate and complex the associations between stink bugs and microorganisms are. Further comprehension of their biological, physiological, and ecological features is necessary to have a better picture of the evolution of these interactions and to devise a more effective pest and disease control programs.

Acknowledgments

The authors would like to thank Jacqueline Robson and Mandy Anhalt for comments and help with the English revision. They also would like to thank the help of Dr. Celia Andrade and Osvaldo Capello from Universidade Estadual de Londrina (UEL), PR, Brazil, with the scan electron microscopy (SEM). Additionally, the authors would like to thank Dr. Jeffrey Aldrich (USDA-ARS) and the 3 anonymous reviewers that revised and positively contributed with this paper.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 536149, 8 pages
doi:10.1155/2012/536149

Research Article

Pheromone of the Banana-Spotting Bug, *Amblypelta lutescens lutescens* Distant (Heteroptera: Coreidae): Identification, Synthesis, and Field Bioassay

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Received 14 February 2012; Accepted 21 April 2012

Academic Editor: Jocelyn G. Millar

The banana-spotting bug, *Amblypelta lutescens lutescens* Distant (Heteroptera: Coreidae), is one of the principal pests of tree fruits and nuts across northern and eastern Australia. Apart from visual damage assessment, there are currently no reliable methods for monitoring bug activity to aid management decisions. An attractant pheromone for this species that could be used as a trap lure could potentially fill this void. Earlier, two male-specific compounds were identified in airborne extracts from *A. lutescens lutescens*, (*E*,*E*)-α-farnesene and (*R*,*E*)-nerolidol; an unknown compound with a molecular weight 220 was also detected. We now report the identification of this hitherto unknown compound as (*R*,*E*,*E*)-α-farnesene-10,11-oxide. Synthesis of this epoxide was conducted using a regioselective asymmetric dihydroxylation of a sulfolene. A blend mimicking the natural proportions of (*E*,*E*)-α-farnesene, (*R*,*E*)-nerolidol, and (*R*,*E*,*E*)-α-farnesene-10,11-oxide attracted male and female *A. lutescens lutescens* as well as nymphs in the field, verifying that the aggregation pheromone comprises or is contained within this group of compounds.

1. Introduction

The fruit-spotting bug, *Amblypelta lutescens lutescens* Distant (Heteroptera: Coreidae), commonly known as the banana-spotting bug, is one of the most important insect pests of fruit and nut crops in tropical and subtropical Australia [1–4]. Nymphs and adults feed on shoots and developing fruits of a wide range of commercial tree crops, causing significant production losses if pesticides are not regularly applied. Monitoring banana-spotting bug activity in crops is extremely difficult and currently relies on scouts assessing damage at weekly or fortnightly intervals throughout the season [5]. This approach is reactive and not always effective, as well as being time consuming and expensive since a large proportion of a crop must be monitored because damage can be unevenly distributed throughout an orchard. Alternative monitoring tools based on semiochemicals, such as host plant volatiles and/or pheromones, are highly desirable because they could potentially be more reliable and easier to standardize. Studying male-specific compounds from Nearctic and Australasian true bugs, Aldrich et al. identified (*E*,*E*)-α-farnesene and (*R*,*E*)-nerolidol from *A. lutescens lutescens*, whereas a third compound with molecular weight 220 remained unknown [6]. Blends of these chemicals could serve as an attractant pheromone for *A. lutescens lutescens* but their biological importance has not been demonstrated, nor has the structure of the unknown compound been elucidated. The aims of this paper were to address both of these issues.

2. Material and Methods

2.1. Insects. *Amblypelta lutescens lutescens* males were hand-collected from a row of *Murraya paniculata* (orange jessamine) bushes on the northwest outskirts of Mareeba, Australia (16°59′S, 145°25′E), at the end of March 2009. Males were separated from females by the squarer shape of the tip of the abdomen when viewed laterally. The collected males were held in a 30 × 30 × 30 cm gauze-covered cage with *M. paniculata* fruit, green beans, and water at approximately 26°C and 60–70% RH under natural light until aeration experiments.

2.2. Collection of Airborne Volatiles from Males. Two samples of airborne extracts were collected in Mareeba from 14 male *A. lutescens lutescens* over a 4- and then a 3-day period in early April 2009. During aerations, the males were held in a 500 mL triple-neck vacuum flask with 3 green beans as food. The flask inlet was connected by silicone tubing (8 mm i.d.) to a modified Pasteur pipette (5 mm i.d.) containing 50 mm of acid-cleansed activated charcoal to filter the incoming air. The flask outlet was also connected by silicone tubing to an 18 cm long glass tube (5 mm i.d.) containing 50 mm of Super Q absorbent (Alltech, USA) held in place on each end by glass wool. Silicone tubing connected the glass tube to an Airchek 224-PCXR8 Sampler pump (SKC Inc., Eight-Four PA), which operated at 250–500 mL/min during the aerations. All connections in the inlet and outlet lines were sealed with Teflon tape. Sampling was conducted at approximately 26°C and 60–70% RH. No bugs died during either sampling period. Once collected, the aeration samples in their glass tubes were sealed in individual airtight containers and sent to USDA-ARS, Beltsville, where they were eluted with 600 μL of hexane. The extracts were concentrated to 50 μL under a gentle stream of N_2 and analyzed by gas chromatography-mass spectrometry (GC-MS) on an Rtx-5MS and by GC on Chiraldex B-DM columns (see below).

2.3. Analytical Methods. Electron-impact (EI) mass spectra were obtained at 70 eV with an Agilent Technologies 5973 mass selective detector interfaced with a 6890 N GC equipped with a 30 m × 0.25 mm i.d. × 0.25 μm film Rtx-5MS (Restek Corporation, Bellefonte, PA, USA) column. Column temperature was maintained at 50°C for 5 min and then raised to 260°C at 10°C/min. Helium was used as a carrier gas at 1 mL/min. Enantioselective GC analyses of nerolidol and racemic and optically active (*E*,*E*)-α-farnesene oxide were performed on an Agilent Technologies 6890 N GC equipped with an FID detector and 30 m × 0.25 mm i.d. × 0.12 μm film Chiraldex B-DM column (Astec, Whippany, NJ, USA). Column temperature was maintained at 100°C for 2 min and then raised to 180°C at 2°C/min. Hydrogen was used as a carrier gas at 1 mL/min. ^{1}H and ^{13}C NMR spectra of compounds **6** and **8** were obtained on a Bruker AVIII-600 MHz spectrometer. ^{1}H NMR spectra of compounds **4** and **10** and ^{1}H-^{1}H COSY of **6** were obtained on a Bruker AV-400 MHz spectrometer. The ^{1}H NMR spectrum of compound **5** was obtained on an Anasazi 90 MHz spectrometer (Indianapolis, IN). Chemical shifts are reported in δ units and referenced to the residual $CDCl_3$ solvent signal. Optical rotations were obtained on a Perkin-Elmer 241 polarimeter with a 1.0 mL cell. Thin layer chromatography analyses were conducted on Whatman AL SIL G/UV plates using 20% ethanol solution of phosphomolybdic acid and/or UV for visualization of spots. Flash chromatography was carried out with 230–400 mesh silica gel (Fisher Scientific, Fair Lawn, NJ).

2.4. Chemicals. All reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI) unless otherwise specified. Hexamethylphosphoramide (HMPA, highly toxic) was distilled from P_2O_5, and tetrahydrofuran (THF) was dried by distillation from sodium benzophenone-ketyl. (*R*,*E*)-Nerolidol was synthesized from (*E*,*E*)-farnesol using a four-step procedure described by Kigoshi et al. [7] that involved a Sharpless-Katsuki asymmetric epoxidation, conversion of the resulting (2*R*,3*R*)-epoxyfarnesol to a tosylate and further to (2*R*,3*R*)-epoxyfarnesyl iodide, and, finally, a stereoselective zinc reduction of the latter. The enantiomeric purity of (*R*,*E*)-nerolidol, as judged from GC analysis on a Chiraldex B-DM column, was 93% (see Section 2.3 for GC conditions). Racemic (*E*,*E*)-α-farnesene oxide (*rac*-**4**) was prepared by epoxidation of (*E*,*E*)-α-farnesene with *m*-chloroperbenzoic acid (MCPBA) as described in Spicer et al. [8].

2.4.1. (E)- 2-(3′,7′-Dimethylocta-2′,6′-dienyl)-3-methyl-2,5-dihydrothiophene-1,1-dioxide (5). Procedures of Yamada et al. [9] and Desai et al. [10] were followed. 3-Methyl-2,5-dihydrothiophene-1,1-dioxide (3-methyl-3-sulfolene, 10.95 g, 82.84 mmol, Alfa Aesar, Ward Hill, MA; recrystallized from methanol before use) was placed under N_2 into a four-neck round bottomed flask equipped with a thermometer, mechanical stirrer, dropping funnel, N_2 inlet, and a septum. Dry THF (300 mL) was added; the solution was cooled to −72°C and then charged sequentially with geranyl bromide (9.0 g, 41.44 mmol) and anhydrous hexamethylphosphoramide (HMPA, 29 mL). Lithium bis(trimethylsilyl)amide (41.4 mmol; 41.4 mL of 1.0 M in THF) was added slowly under vigorous stirring, maintaining the temperature below −68°C. The reaction mixture was stirred for 30 min at −70°C, then slowly warmed to −20°C, and quenched with ethyl acetate (150 mL). The mixture was concentrated on a rotary evaporator, and the residue was dissolved in ether (500 mL), washed with water and brine, and then dried over anhydrous sodium sulfate. Evaporation of the solvent and flash chromatography of the residue with hexane/ethyl acetate (3 : 1) yielded compound **5** (9.30 g, 84% from geranyl bromide). GC-MS analysis of **5** proceeded with complete extrusion of SO_2 as described in Chou et al. [11] resulting in (*E*,*E*)-α-farnesene of 95% purity. GC-MS (*m*/*z*, %): 204 (M^+, 1), 189 (2), 161 (5), 133 (5), 123 (33), 119 (38), 107 (46), 105 (24), 93 (100), 91 (38), 81 923), 80 (24), 79 (45), 77 (28), 69 (58), 67 (12), 55 (50), 53 (17), 43 (16), 41 (69). The mass spectral data matched those reported in NIST 08 MS Library for (*E*,*E*)-α-farnesene. ^{1}H NMR (90 MHz, $CDCl_3$): 1.58 (br s, 3H), 1.62 (br s, 6H), 1.82 (m, 3H), 1.95–2.06 (m, 4H), 2.57 (br t, J = 7.0 Hz, 2H), 3.38–3.70 (m, 3H), 5.06 (m, 1H), 5.20 (br t, J = 7.0 Hz, 1H), 5.62 (m, 1H). Data are in good agreement with those reported in [8].

2.4.2. (3E,6E)-3,7,11-Trimethyl-1,3,6,10-dodecatetraene ((E,E)-α-Farnesene 2). (*E,E*)-α-Farnesene was prepared by thermal decomposition of **5** (100 mg, 0.37 mmol) in degassed refluxing *n*-octane (5 mL) bubbled with N_2 for 1.5 h [12]. The solution was cooled, concentrated, and purified by flash chromatography with pentane to give **2** (61 mg, 81%) of 94% purity.

2.4.3. (2R/S,6′S,2′E)- 2-(3′,7′-Dimethyl-6′,7′-dihydroxy-2′-octenyl)-3-methyl-2,5-dihydrothiophene-1,1-dioxide (6). A procedure by Sharpless et al. [13] was followed. AD-mix α (26.0 g) was added to a stirred mixture of *t*-BuOH (83 mL) and water (93 mL) at 0–5°C. Methanesulfonamide (1.70 g, 17.90 mmol) was added, followed by sulfolene **5** (5.0 g, 18.62 mmol). The mixture was stirred for 8 h, after which TLC analysis (hexane/ethyl acetate, 1 : 3) showed very little remaining starting material. Sodium sulfite (2.35 g) was added, and the mixture was allowed to warm to room temperature. The mixture was diluted with water (40 mL) and extracted with dichloromethane (6 × 50 mL). The organic extracts were combined, dried with Na_2SO_4, and concentrated. Flash chromatography with hexane/ethyl acetate (1 : 3) provided diol **6** (4.96 g, 88%) as a mixture of two diastereomers in about equal quantity. GC-MS (*m/z*, %): 179 (3), 159 (3), 134 (28), 121 (24), 119 (55), 107 (28), 93 (66), 91 (40), 81 (67), 80 (58), 79 (47), 59 (100), 43 (47), 41 (42). $[\alpha]_D^{25} = -9.26°$ (*c* 1.75, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): 1.149 and 1.154 (s, 3H, C7′-CH_3 A), 1.19 and 1.20 (s, 3H, C7′-CH_3 B), 1.44 (m, 1H, H-5′), 1.62 (m, 1H, H-5′), 1.68 and 1.69 (s, 3H, C3′-CH_3), 1.86 (br s, 3H, C3-CH_3), 2.13–2.28 (m, 4H, H-4′, 2OH), 2.57 (m, 2H, H-1′), 3.35 (t, J = 10.2 Hz, 1H, H-6′), 3.55–3.75 (m, 3H, H-2, H-5), 5.29 and 5.33 (br t, J = 6.5 Hz, 1H, H-2′), 5.69 (br s, 3H, H-4). ^{13}C NMR (151 MHz, $CDCl_3$): 15.97, 16.12, 17.95, 18.02, 23.36, 23.39, 25.98, 26.00, 26.27, 26.30, 28.61, 29.17, 36.52, 36.67, 43.40, 55.73, 67.25, 67.44, 72.96, 77.52, 117.16, 117.18, 119.04, 119.27, 138.26, 138.33, 138.69, 138.80.

2.4.4. (2R/S,6′R,2′E)- 2-(3′,7′-Dimethyl-6′,7′-dihydroxy-2′-octenyl)-3-methyl-2,5-dihydrothiophene-1,1-dioxide (7). Analogous to the procedure described above, sulfolene **5** (1.498 g, 5.58 mmol) was dihydroxylated with AD-mix β (7.79 g) in the presence of methanesulfonamide (0.53 g, 5.58 mmol) in a mixture of *t*-BuOH (27 mL) and water (27 mL) to give diol **7** (1.200 g, 71%). $[\alpha]_D^{25} = +10.49°$ (*c* 2.64, $CHCl_3$).

2.4.5. (3S,6E,9E)-2,6,10-trimethyl-6,9,11-dodecatriene-2,3-diol (8). A two-neck conical flask equipped with a reflux condenser and N_2 inlet extending to the bottom of the flask was loaded with a mixture of sulfolenediol **6** (491 mg, 1.62 mmol) and pyridine (10 mL). The solution was degassed with a gentle steam of N_2 for 10 min and then heated in an oil bath at 122°C while maintaining the N_2 flow. After 2 h of heating, TLC (hexane/ethyl acetate, 1 : 3) showed that the reaction was almost complete. The solution was concentrated by rotary evaporation, and the remainder was purified by flash chromatography with (hexane/ethyl acetate, 1 : 1) to provide diol **8** (287 mg, 74%). GC-MS (*m/z*, %): 205 (1), 179 (5), 159 (5), 143 (7), 134 (40), 121 (32), 119 (71), 107 (33), 105 (37), 93 (72), 91 (43), 81 (74), 80 (66), 79 (48), 59 (100), 43 (31), 41 (26). $[\alpha]_D^{25} = -23.9°$ (*c* 2.70, $CHCl_3$). Lit. [14] $[\alpha]_D = -20.8°$ (*c* 0.03, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): 1.16 (s, H-1), 1.21 (s, 3H, C2-CH_3), 1.60 (m, 2H, H-4), 1.66 (s, 3H), 1.77 (s, 3H), 2.09 (m, 1H, H-5A), 2.27 (m, 1H, H-5B), 2.86 (t, J = 7.2 Hz, 2H, H-8), 3.36 (m, 1H, H-3), 4.94 (d, J = 10.8 Hz, 1H, H-12 *cis*), 5.10 (d, J = 17.4 Hz, 1H, H-12 *trans*), 5.20 (t, J = 6.0 Hz, 1H, H-7), 5.45 (t, J = 7.5 Hz, 1H, H-9), 6.36 (dd, J = 17.4, 10.8 Hz, 1H, H-11). ^{13}C NMR (151 MHz, $CDCl_3$): 11.9, 16.3, 23.31, 26.7, 27.4, 29.9, 36.9, 73.3, 78.5, 110.9, 122.9, 131.6, 134.1, 135.9, 141.7. ^{1}H NMR data are in close agreement with reported values [14].

2.4.6. (3R,6E,9E)-2,6,10-Trimethyl-6,9,11-dodecatriene-2,3-diol (9). Analogously to the preparation of diol **8**, sulfolene-diol **7** (1.250 g, 4.13 mmol) was heated in the presence of pyridine (50 mL) to provide diol **9** (546 mg, 55%, 98% pure). The mass spectrum of **9** was identical to that of **8**. $[\alpha]_D^{25} = +25.0°$ (*c* 2.58, $CHCl_3$). Lit. [14] $[\alpha]_D = +21.2°$ (*c* 0.05, $CHCl_3$).

2.4.7. (10R,3E,6E)-3,7,11-Trimethyl-10,11-epoxy-1,3,6-dodecatriene ((R,E,E)-α-farnesene-10,11-oxide, 4). Methanesulfonyl chloride (138 μL, 1.73 mmol) was added to a solution of diol **8** (378 mg, 1.59 mmol) in dry pyridine (2 mL) at room temperature. After stirring for 1 h, TLC (hexane/ethyl acetate, 1 : 1) confirmed a complete conversion of the diol to the secondary mesylate. The mixture was cooled in an ice bath and quenched with a mixture of water (3 mL) and methyl *t*-butyl ether (3 mL). The organic layer was separated, and the aqueous layer was extracted with methyl *t*-butyl ether (5 × 3 mL). The organic extracts were combined, dried over Na_2SO_4, and concentrated. The crude mesylate (still containing pyridine) was dissolved in methanol (5 mL) and treated with a solution of KOH (162 mg, 2.89 mmol) in MeOH (1.5 mL) at 20°C. A precipitate formed almost instantaneously, and an additional 3 mL of MeOH was added to enable stirring for another 30 min. The methanol then was removed *in vacuo*, and the residue was partitioned between ice water (3 mL) and ether (5 mL). The organic layer was separated, and the aqueous layer was extracted with hexane/ether (1 : 1). The combined organic extracts were washed with ammonium chloride solution, dried, and concentrated. The remainder was purified by flash chromatography (hexane/ethyl acetate, 1 : 1) to provide epoxide **4** (223 mg, 64%) of 93% chemical and 95% enantiomeric purities. The latter was determined by chiral GC analysis on the Chiraldex B-DM column (see Section 2.3 for conditions). $[\alpha]_D^{25} = +0.2°$ (*c* 1.45, $CHCl_3$). GC-MS (*m/z*, %): 205 (1), 187 (2), 159 (4), 134 (40), 119 (100), 107 (26), 105 (45), 93 (83), 91 (63), 81 (57), 80 (75), 79 (70), 77 (39), 59 (43), 55 (44), 43 (56), 41 (61). The mass spectral data matched those of the natural epoxide **4** and synthetic racemic epoxide [15]. ^{1}H NMR (400 MHz, $CDCl_3$): 1.26 (s, 3H, H-12/C11-CH_3), 1.30 (s, 3H, H-12/C11-CH_3), 1.67 (br s, 3H), 1.58–1.75 (m, 2H), 1.77 (br s, 3H), 2.07–2.22 (m, 2H), 2.71 (t, J = 6.0 Hz, 1H, H-10), 2.85 (br t, J = 7.2 Hz, 2H, H-5),

4.94 (d, J = 10.8 Hz, 1H, H-1 *cis*), 5.10 (d, J = 17.6 Hz, 1H, H-1 *trans*), 5.18 (dq, J = 7.2, 1.2 Hz, 1H), 5.46 (br t, J = 7.2, 1H), 6.37 (dd, J = 17.6, 10.8 Hz, 1H, H-2). These data are in good agreement with those previously reported for the racemic epoxide [8, 15].

2.4.8. (10S,3E,6E)-3,7,11-Trimethyl-10,11-epoxy-1,3,6-dodecatriene ((S,E,E)-α-farnesene oxide, 10). Diol **9** (490 mg, 2.06 mmol) was mesylated with methanesulfonyl chloride (179 μL, 2.25 mmol) in dry pyridine (2.5 mL) as described above, and the crude mesylate was treated with KOH (233 mg) in 10 mL MeOH to yield epoxide **10** (222 mg, 50%) of 92% of chemical and 94% enantiomeric purities. $[\alpha]_D{}^{25}$ = −0.1° (c 2.50, $CHCl_3$). The mass spectral and NMR data of **10** were identical to those of **4**.

2.5. Field Trapping. Two treatments were tested in a mango orchard on Southedge Research Station, approximately 50 km west of Cairns in northeast Queensland, Australia: no.1, 0.2 mg (*E*,*E*)-α-farnesene (**2**), 1.2 mg (*R*,*E*)-nerolidol (**3**), and 0.6 mg (*R*,*E*,*E*)-α-farnesene oxide (**4**); no. 2, 0.2 mg (*E*,*E*)-α-farnesene, 1.2 mg (*R*,*E*)-nerolidol, 0.6 mg (*R*,*E*,*E*)-α-farnesene oxide, and 0.6 mg hexyl hexanoate (**1**). Relative proportions of components in these blends were based on relative areas of peaks in the aeration extract. The pheromone components plus 5% BHT (butylated hydroxytoluene, or 2,6-di-*tert*-butyl-4-methylphenol) stabilizer were applied to rubber septa in hexane solutions [16] at Beltsville and shipped to Australia. The test site was located at 16°58′S, 145°20′E at an elevation 450 m, and has a mean annual rainfall of 1,110 mm. The station has several thousand mango trees, including an extensive gene pool block, mixed varietal block, a large number of hybrids, and pure blocks containing Kensington Pride, R2E2, and Keitt varieties. The traps were set out in two rows of mature trees (16–20 years old) of mixed varieties at one trap per tree with several trees without traps between them. All trees contained maturing fruit at the time of trap deployment in October. Traps consisted of panels of green-colored twin wall polypropylene sheeting (Corflute BFS Plastic Pty. Ltd., Salisbury, QLD, Australia, 0.3 × 25 × 30 cm), placed 2–2.5 m above ground and fixed to branches with wire so that they remained rigid even in windy conditions. Traps were covered on each side with double-sided cloth tape coated with emulsion acrylic adhesive (Henkel Aust. Pty. Ltd., Kilsyth, VIC, Australia). Each trap had a 3 cm diameter hole in the center in which the rubber septum containing the pheromone was fastened. There were five replicates of each pheromone treatment and five untreated control traps. Traps were assigned sequentially for treatments, repeated for each subsequent replicate down the rows. Pheromone lures were stored, sealed, and refrigerated until use, and traps were baited the morning of deployment. Traps were inspected 4 days after their initial deployment, when adult males, females, and nymphs were removed and recorded.

2.6. Analysis of Trapping Data. The data are presented as number of *A. lutescens lutescens* adult males, adult females, and nymphs per trap per day. Data were $\sqrt{(x+1)}$-transformed to compare treatments by analysis of variance using GenStat 11th Edition [17]. Significantly different means were compared by the least significant difference method at P = 0.05.

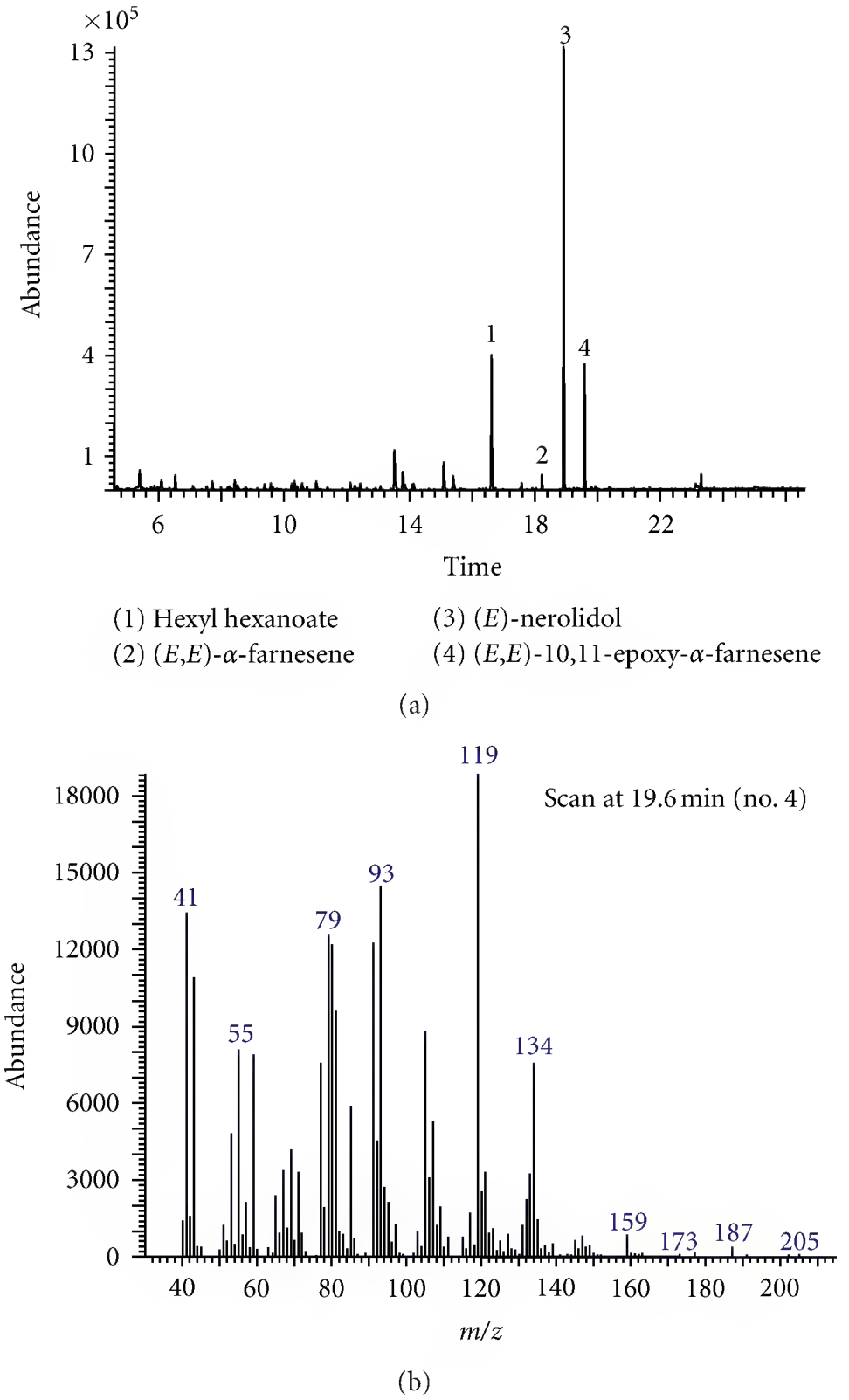


FIGURE 1: Total ion chromatogram of an aeration extract of male *Amblypelta lutescens lutescens* and EI mass spectrum of compound **4**. Column: Rtx-5MS (30 m × 0.25 mm i.d. × 0.25 μm), 50°C (5 min) to 260°C at 10°C/min.

3. Results and Discussion

3.1. Identification and Synthesis. The total ion chromatogram (Figure 1) revealed several compounds, among which were the previously identified (*E*,*E*)-α-farnesene (**2**) and (*E*)-nerolidol (**3**) [6], plus compound **4**, the mass spectrum of which matched that of the unknown male-specific compound showing a molecular weight 220, previously found by Aldrich et al. [6]. In addition to these compounds, in the aeration sample from males we found a relatively large amount of hexyl hexanoate (**1**), a trace of which was

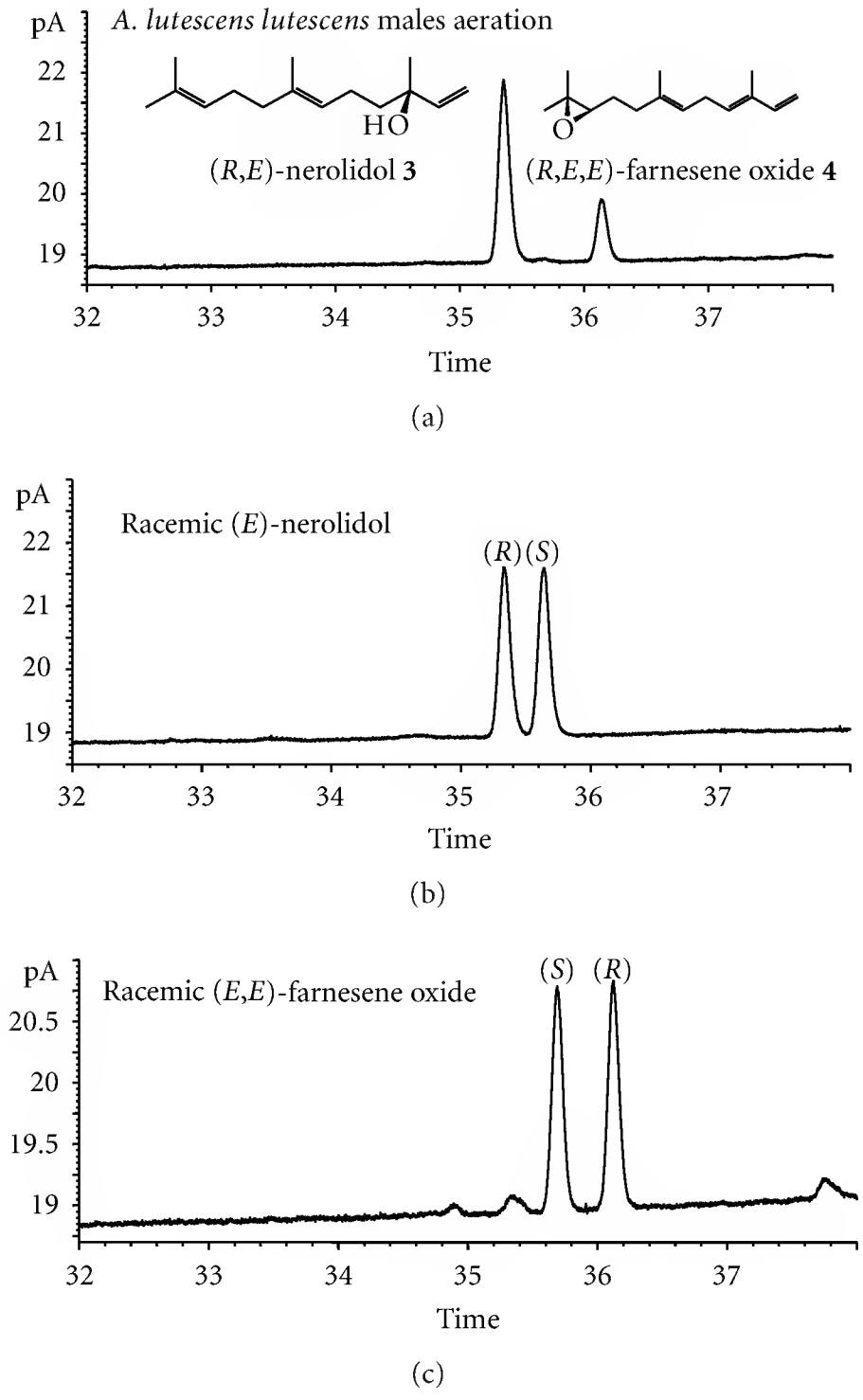
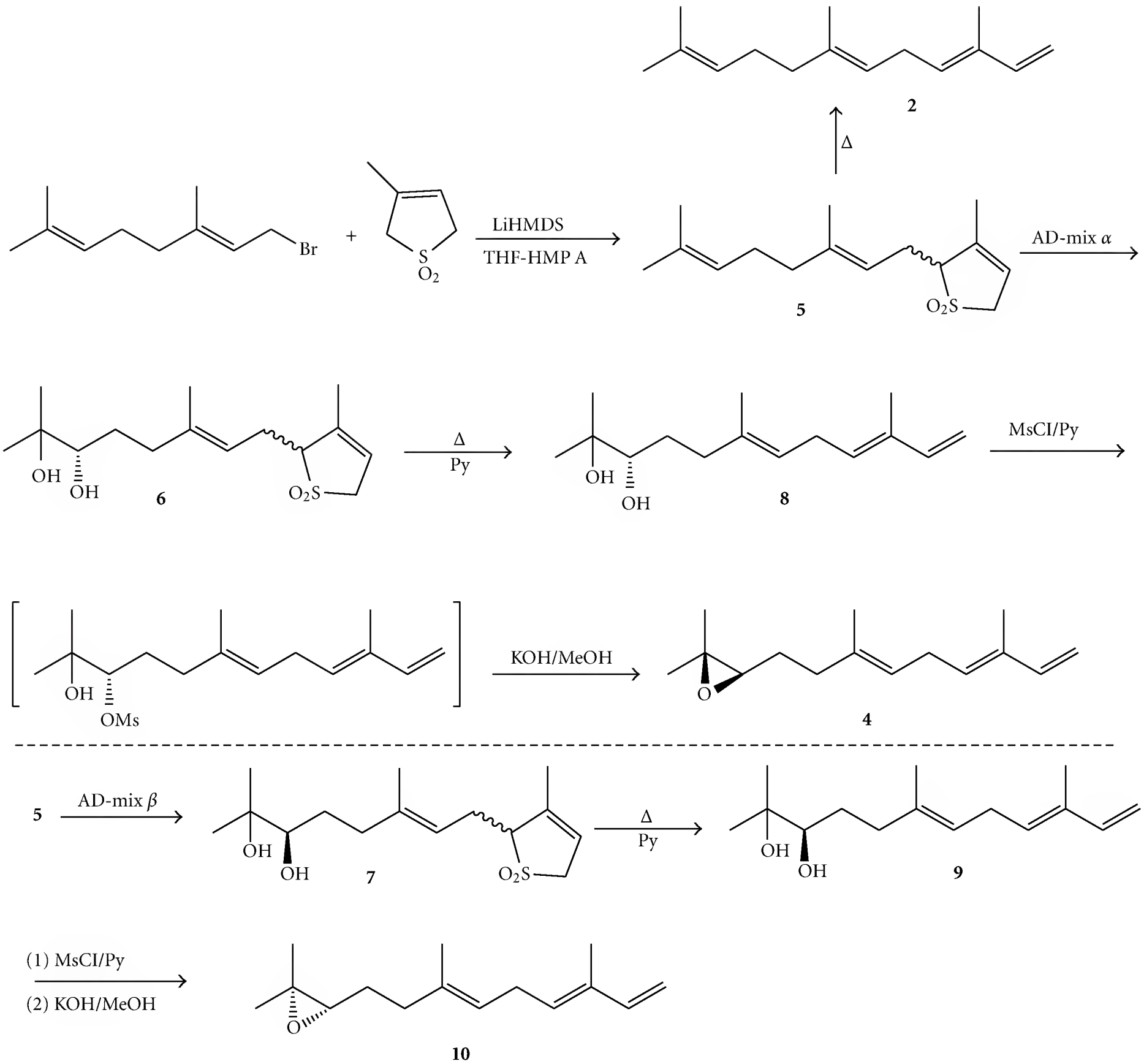


FIGURE 2: Sections of GC-FID traces of an aeration extract of male *Amblypelta lutescens lutescens* (a), racemic (*E*)-nerolidol (b), and racemic (*E*,*E*)-farnesene oxide (c). Column: Chiraldex B-DM (30 m × 0.25 mm i.d. × 0.12 μm), 100°C (2 min) to 180°C at 2°C/min.

previously found in female but not male *A. lutescens lutescens* [6]. Because the unidentified compound **4** conceivably could have been an epoxide of (*E*,*E*)-α-farnesene, we decided to start with a random epoxidation of (*E*,*E*)-α-farnesene that had already been reported [8, 15]. We repeated the *m*-chloroperbenzoic acid epoxidation of (*E*,*E*)-α-farnesene in methylene chloride and found that the monoepoxidation product at the 10,11 double bond matched compound **4** by GC retention time and mass spectrum. The Chiraldex B-DM GC column resolved (Figure 2) synthetic racemic farnesene oxide **4** (lower chromatogram) and racemic (*E*)-nerolidol to baseline (middle chromatogram). Analysis of the *A. lutescens lutescens* aeration sample of males on the Chiraldex B-DM column (upper chromatogram) revealed that only one enantiomer of (*E*)-nerolidol and farnesene oxide were produced by the bug. Aldrich et al. [6] had already determined that (*E*)-nerolidol produced by *A. lutescens lutescens* had the (*R*)-configuration, and we confirmed this assignment by showing that synthetic (*R*,*E*)-nerolidol had the same GC retention time as the natural compound, or the first eluting enantiomer of racemic (*E*)-nerolidol.

Determination of the enantiomeric composition of (*E*,*E*)-α-farnesene-10,11-epoxide **4** produced by *A. lutescens lutescens* necessitated synthesis of both enantiomers. Thus, we considered a route developed by Moore et al. [18] for synthesis of ocimene epoxides, which was based on a Sharpless asymmetric dihydroxylation and further stereoselective cyclization of intermediate chiral diols to epoxides through secondary mesylates. In fact, asymmetric dihydroxylation of (*E*,*E*)-α-farnesene **2** had been reported in the literature [14] but only in very low yields (11 and 15% for (−) and (+) diols, resp.) for a 10,11-dihydroxylation product. The low yield of the desired diol was a reflection of the multiple sites of unsaturation present in (*E*,*E*)-α-farnesene that were susceptible to asymmetric dihydroxylation. To overcome this shortcoming, we developed a new route presented in Scheme 1, which was essentially an enantioselective version of the synthesis of racemic (*E*,*E*)-α-farnesene-10,11-epoxide developed by Fielder and Rowan [15]. The synthesis started with alkylation of 3-methyl-3-sulfolene with geranyl bromide, as described in several reports (see Section 2). The key step, asymmetric dihydroxylation of the intermediate **5** with AD-mix α, was highly regioselective (as was MCPBA epoxidation of **5** [15]) and offered sulfolenediol **6** in 88% yield. ^{1}H NMR spectrum of diol **6** recorded at 600 MHz clearly shows the presence of two diastereomers with separation of several signals, including $(CH_3)_2COH$, even though the two chiral centers are separated by five carbon atoms (see Section 2). The observed regioselectivity of asymmetric dihydroxylation of **5** was in stark contrast with direct asymmetric dihydroxylation of (*E*,*E*)-α-farnesene [14] and was similar to the analogous dihydroxylation of ocimene [18] and squalene [19], whereby the reactions occurred primarily at the terminal trisubstituted double bonds.

Thermal elimination of sulfur dioxide in **6** was accomplished in pyridine [10] to minimize side reactions and polymerization due to the acidity of SO_2. For the same reason, mesylation of diol **8** was also conducted in pyridine, and in the last step, the crude mesylate was cyclized to epoxide **4** with potassium hydroxide in methanol [20]. Analogously, sulfolene **5** was dihydroxylated with AD-mix β to provide diol **7**, which was then thermally transformed to diol **9**. Cyclization of the latter as described above furnished enantiomeric farnesene epoxide **10**. Both **4** and **10** were prepared in ≥94% ee as judged by GC analysis on the Chiraldex B-DM column, which indicated that asymmetric dihydroxylation proceeded with high enantioselectivity, as anticipated from the original report by Sharpless et al. [13] and the subsequent results of Moore et al. [18]. High optical purities of epoxides **4** and **10** were also consistent with previous reports [18, 20] and indicative of highly stereoselective, base-catalyzed S_Ni substitution in the cyclization step. Overall, the route capitalizes on the much higher regioselectivity of dihydroxylation of sulfolene **5** compared to direct dihydroxylation of (*E*,*E*)-α-farnesene and, in fact, does not add additional steps to the synthesis of diols **8** and **9** (and epoxides **4** and **10**) because one of the best known synthetic routes to (*E*,*E*)-α-farnesene includes intermediate **5** [11].



Scheme 1: Syntheses of (*R*,*E*,*E*)- and (*S*,*E*,*E*)-α-farnesene-10, 11-oxides **4** and **10**.

With both enantiomers of farnesene epoxide **4** and **10** available, we identified the later eluting peak in the gas chromatogram of the racemic epoxide on Chiraldex B-DM (Figure 2, lower chromatogram) as the (*R*)-enantiomer and, hence, determined that the natural farnesene epoxide (Figure 2, upper chromatogram) collected from *A. lutescens lutescens* had the (*R*)-configuration.

3.2. Field Bioassay. In preliminary trapping experiments pheromone lures were deployed on painted plywood panels covered with Tangle-Trap insect barrier gel (The Tanglefoot Co., Grand Rapids, MI, USA). When deployed in avocado and mango orchards, these traps baited with either the 3- or 4-component pheromone lures (see Section 2) only caught 0.01-0.02 *A. lutescens lutescens*/trap/day. It was observed that many bugs attracted to the pheromone lures were able to escape from the traps unless caught on their backs or sides.

The Corflute panels covered by adhesive tape were much more effective at trapping *A. lutescens lutescens* and, when deployed with the 3- and 4-component pheromone lures in mangoes, caught up to 0.90 ± 0.23 fruit-spotting bugs/trap/day (mean ± SE) (Table 1). When run in the same trial, the plywood panels with a tangle-trap gel caught a maximum of 0.10 ± 0.06 fruit-spotting bugs/trap/day.

There was no statistical difference in the total numbers of *A. lutescens lutescens* attracted to either the 3- or 4-component lures, and traps baited with both caught significantly more bugs than the untreated control traps (Table 1). Thus, hexyl hexanoate does not seem to be a component of the attractant pheromone for *A. lutescens lutescens*. Both pheromone treatments attracted adult males, adult females, and nymphs, without a clear dominance of one sex or stage over another. Hence, our results indicate that the blend of male-specific compounds **2–4** found in the male *A. lutescens*

Table 1: Mean numbers of *A mblypelta lutescens lutescens* trapped in a mango crop in north Queensland by sticky panels containing 3- and 4-component pheromone lures over a 4-day period in October 2011.

Panel traps	Mean no. of *A. lutescens lutescens*/trap/day (±SE)				Total bugs caught
	Adults		Nymphs	Total	
	♂♂	♀♀			
3-component lure	0.45 (±0.16) a	0.25 (±0.08) a	0.20 (±0.09) a	0.90 (±0.23) a	18
4-component lure	0.15 (±0.10) ab	0.20 (±0.05) a	0.10 (±0.06) ab	0.45 (±0.12) a	9
Untreated control	0 b	0 b	0 b	0 b	0

Means within columns followed by the same letter are not significantly different at $P = 0.05$.

lutescens aerations contained the aggregation pheromone for this species. Whereas (*R*,*E*)-nerolidol had been identified previously from at least two bug species [6] and (*E*,*E*)-α-farnesene was recently identified as a termite alarm pheromone [21], to our knowledge, (*R*,*E*,*E*)-α-farnesene-10,11-oxide **4** has not been reported as a pheromone component for any other insect species. More research is needed to determine whether the actual pheromone consists of one, two, or all three of these compounds, and for practical purposes, the optimal dose(s) and importance of chirality of the farnesene oxide must be determined. Preliminary experiments have indicated that doubling or tripling the emission rate of the 3-component lure as used above did not increase bug catches. The recent deregistration of endosulfan in Australia has significantly reduced the insecticidal options for the control of *A. lutescens lutescens*, and an effective monitoring tool based on a pheromone lure may become an essential tool in the management of the banana-spotting bug.

Acknowledgments

Geoff Waite, now retired from DEEDI, collaborated in much of the earlier work on *Amblypelta* pheromones and should be acknowledged for his contributions in this area. Robert Bauer (DEEDI) assisted with the field trials of the pheromones in north Queensland and the Australian Centre for International Agricultural Research provided funds in support of this work.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 470436, 16 pages
doi:10.1155/2012/470436

Review Article

An Insight into the Sialomes of Bloodsucking Heteroptera

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Received 27 January 2012; Accepted 17 April 2012

Academic Editor: Mark M. Feldlaufer

Saliva of bloodsucking arthropods contains dozens or hundreds of proteins that affect their hosts' mechanisms against blood loss (hemostasis) and inflammation. Because acquisition of the hematophagous habit evolved independently in several arthropod orders and at least twice within the true bugs, there is a convergent evolutionary scenario that creates a different salivary potion for each organism evolving independently to hematophagy. Additionally, the immune pressure posed by their hosts creates additional evolutionary pressure on the genes coding for salivary proteins, including gene obsolescence, which opens the niche for coopting new genes (exaptation). In the past 10 years, several salivary transcriptomes from bloodsucking Heteroptera and one from a seed-feeding Pentatomorpha were produced, allowing insight into the salivary potion of these organisms and the evolutionary pathway to the blood-feeding mode.

1. Introduction

The order Hemiptera (bugs) comprises hemimetabolous insects having in common tubular mouthparts specialized for sucking liquid diets. The diet of Hemiptera is varied, the majority feeding on plants by either tapping the vessels conducting sap or by lacerating and flushing tissues such as leaves or seeds. Within the suborder Heteroptera (true bugs), predatory feeding (with killing of the victim) also occurs, mostly targeting other insects but also including small vertebrates such as giant water bugs and toad bugs, as well as blood or hemolymph feeding (without killing the victim) from vertebrate and invertebrate animals. The mouthparts are not only important for channeling the liquid meal but are extremely important mechanically in finding the proper spot for meal suction [1].

Saliva is produced, sometimes copiously, during the probing phase (the time between mouthpart contact with the food substrate and the commencement of the meal) and throughout the meal [31, 32]. This saliva is ejected at the tip of the maxillae by the salivary channel, which is built in between the interdigitations of the two plates that form the maxillae [33]. Saliva helps probing and feeding physically by liquefying insoluble or viscous tissues or by helping to seal the feeding site in sap suckers, were the phloem is under very high pressure [34]. Saliva has a biochemical role in aiding digestion of the meal, just as we have amylase in our own saliva; most remarkably, predacious bugs inject a highly hydrolytic cocktail into their victims that is digested while the prey is held by the predator, which can then later suck the liquefied victim and discard it as an empty shell. Saliva can also work pharmacologically by preventing the hosts' defense mechanisms against tissue loss, as occurs with the saliva of blood-feeding insects in preventing blood clotting, for example [35].

Among the Heteroptera, the blood-feeding habit evolved at least twice in the Cimicomorpha families, once in the Cimicidae (containing the bed bug) including the small sister group Polyctenidae (bat bugs), and in the Reduviidae (kissing bugs) from possible predacious or hemolymph-sucking ancestors [1]. Within the Reduviidae, it is possible that the genus *Rhodnius* (tribe Rhodnini) is monophyletic, having evolved independently of the remaining triatomines (tribe Triatomini) [47–49]. The ancestral Cimicomorpha dates back to the Triassic/Jurassic border, over 250 MYA [1]; accordingly, the habit of blood or hemolymph feeding started

in this group well before mammals irradiated. Within these hematophagous bugs, blood is the only diet for all immature and adult stages.

To obtain blood in a fluid state, these bugs have to counteract their host's hemostasis, the physiologic process that prevents blood loss, which includes the triad of platelet aggregation, blood clotting, and vasoconstriction. Blood-circulating platelets may be triggered to aggregate by various signals, including ADP from broken cells and also released by activated platelets, collagen from subendothelial surfaces, thrombin (produced during blood clotting), and thromboxane A_2 (TXA_2—produced by activated platelets). Blood clotting may be initiated by activation of the intrinsic pathway via activation of Factor XII or by activation of the tissue factor pathway, both converging to the activation of Factor X to X_a, which activates prothrombin to thrombin which in turn cleaves fibrinogen into fibrin, forming the blood clot. Activated platelets produce the vasoconstrictor TXA_2 and also release stored serotonin and epinephrine, both powerful vasoconstrictors. A single magic bullet cannot properly destroy a redundant and complex obstacle such as this; rather, a magic potion of several antagonists is required.

Saliva of hematophagous arthropods also contains activities that interfere with the host's immune and inflammatory system in the form of immunomodulatory substances, particularly in ticks, which stay attached to their hosts for days or weeks, in contrast to minutes of host contact by bloodsucking bugs. Saliva also contains antimicrobial compounds that might help to control bacterial growth in the meal, because ejected saliva is reingested with the blood meal during blood feeding. For more detailed reviews on host hemostasis and immunity, see Francischetti et al. [56, 57].

Salivary anticlotting compounds from bloodsucking insects have been known to occur for nearly 100 years [58], while antiplatelet activity was first detected in the 1980s [59, 60] and vasodilators have only been described since the early 1990s [61]. In blood-feeding bugs, anticlotting agents from the saliva and crop of *Rhodnius* were described first by Hellman and Hawkins in 1965 [62], the first antiplatelet activity was reported in 1981 [59, 63], and *Rhodnius* salivary vasodilator was reported in 1990 [64]. Anticomplement activities have also been found [65], as well as antihistamine, antiserotonin [66], and antithromboxane [67] activities from *Rhodnius* saliva. An anesthetic was found in *Triatoma infestans* saliva in 1999 [68]. None of these earlier reports characterized the molecular nature of the compounds, most of these have been achieved in the past 20 years during the so-called "grind and find" period of discovery. Table 1 lists the molecularly characterized salivary components of blood-feeding Hemiptera.

2. On Sialomes

In the past 10 years, a new method to unveil the salivary potion of hematophagous insects has been practiced in the form of decoding their sialotranscriptomes (from the Greek, sialo = saliva), achieved by random sequencing of 500–2,000 cDNA clones originating from polyA-enriched RNA from the salivary glands of these animals. After assembly of these sequences into contigs (which represent full or near full-length mRNA), these can be compared by bioinformatic tools such as BLAST and rpsblast [69] to other proteins in public databases (such as Swissprot, Gene Ontology [70], and GenBank [71] protein data banks, and CDD, PFAM, SMART and KOG [72], which are motif databases to be explored with the rpsblast tool) to identify closely related sequences and functional motifs. Additional searches for signal sequences indicative of secretion [73], for transmembrane helices [74], and for glycosylation sites [75] are also helpful to attempt functional classification of the protein. We are now on the eve of another revolution, with the increase by thousands of fold on the number of sequences that can be economically sequenced from these libraries, which will allow identification of the lesser expressed (and possibly most potent) proteins.

So far, 12 sialotranscriptomes—all done with less than 3,000 sequenced clones per organism—have been reported from Heteroptera, 11 of which are from blood-feeding Cimicomorpha and one from the seed-feeding *Oncopeltus fasciatus*. *Oncopeltus* belongs to the Pentatomomorpha, the closest group to Cimicomorpha [76] (Table 2). Among the Cimicomorpha sialotranscriptomes, only one derives from Cimicidae (the bed bug *Cimex lectularius*); the remaining are from Triatominae, encompassing four genera (*Rhodnius, Triatoma, Dipetalogaster,* and *Panstrongylus*), although some of these transcriptomes have no proteins deposited in public databases and too few expressed sequence tags (ESTs) publicly available. A few isolated protein sequences are also available from GenBank, deriving mostly from predatory bugs. The publicly available proteins are displayed together in Additional File 1, which is a hyperlinked Excel spreadsheet where the putative secreted proteins are organized in one worksheet and the putative housekeeping proteins are displayed in another worksheet.

The secreted proteins can be classified in two major groups, those belonging to ubiquitous protein families and those of unique status among the Hemiptera family, genus, or even species level (Table 3). We will proceed to describe the protein families in the order shown on Table 3.

3. Ubiquitous Protein Families

3.1. Enzymes

3.1.1. Apyrase, 5′-Nucleotidase, and NUDIX Hydrolase. Apyrases are enzymes that can hydrolyze ATP and ADP to AMP [77–79]. Initially the existence of true apyrases was doubted, because they could originate from a mixture of enzymes such as adenylate kinase and ATPases; however, their real intracellular existence in the potato was shown later [79, 80] and its function in carbohydrate anabolism and in the promotion of glycosyltransferases was only much later discovered, as indicated [81, 82]. The role of extracellular apyrases on preventing platelet aggregation was demonstrated for the first time in *Rhodnius* saliva [63, 83, 84] and later shown in the saliva of mosquitoes [85–87] and in the vascular endothelium [88–90]. The activity from *Cimex lectularius* was purified and cloned, revealing a new type of

Table 1: Molecularly and functionally characterized salivary components of bloodfeeding Hemiptera.

Name	Family	Insect	Activity	Notes	Reference
Prolixin	Nitrophorin	*Rhodnius prolixus*	Anticlotting	FXa inhibitor	[2]
RPAI	Lipocalin	*R. prolixus*	Antiplatelet	Binds ADP	[3, 4]
Nitrophorins	Nitrophorin	*R. prolixus*	Antihistamine	Binds histamine, carrier of NO	[5, 6]
BABP	Nitrophorin	*R. prolixus*	Antiserotonin	Binds serotonin	[7]
Inositol phosphatase	Inositol phosphatase	*R. prolixus*	Inositol phosphatase	Unknown function	[8]
Lysophosphatidylcholine	Lipid	*R. prolixus*	Antihemostatic		[9]
NO	Inorganic gas	*R. prolixus, Cimex lectularius*	Vasodilatory, antiplatelet	Activates guanylate cyclase	[10, 11]
Apyrase	5′-nucleotidase	*Triatoma infestans*	Antiplatelet	Destroys ADP	[12, 13]
Triplatin	Lipocalin	*T. infestans*	Antiplatelet, vasodilator	TXA_2 binder	[14, 15]
Triafestin	Lipocalin	*T. infestans*	Anticlotting, antipain	Inhibits FXII activation	[16]
Trialysin	Trialysin	*T. infestans*	Antimicrobial	Pore forming	[17, 18]
Pallidipin	Lipocalin	*T. pallidipennis*	Antiplatelet	Collagen inhibitor (possible TXA_2 binder)	[19, 20]
Triabin	Lipocalin	*T. pallidipennis*	Anticlotting	Anti-thrombin	[21–23]
Procalin	Lipocalin	*T. protracta*	Allergen	Function unknown	[24]
Dipetalodipin	Lipocalin	*Dipetalogaster maxima*	Antiplatelet, vasodilator	TXA_2 binder	[25]
Apyrase	*Cimex* apyrase	*Cimex lectularius*	Antiplatelet	Destroys ADP	[26]
Nitrophorin	Inositol phosphatase	*C. lectularius*	Antiplatelet, vasodilator	Carrier of NO	[27–29]
Fibrinolytic enzyme	Serine proteinase	*Panstrongylus megistus*	Anticlotting		[30]

Table 2: Salivary transcriptomes of Hemiptera/Heteroptera.

Organism	Number of ESTs on DBEST	Number of derived proteins in GenBank	Reference
Rhodnius prolixus	1,439	56	[36]
R. brethesi	55	0	[37]
R. robustus	121	0	[37]
Triatoma infestans	1,738	167	[38]
Triatoma brasiliensis	2,109	28	[39]
Triatoma matogrossensis	2,230	196	[40]
Triatoma rubida	1,850	93	[41]
Triatoma dimidiata	53	53	[42]
Dipetalogaster maxima	2,671	66	[43]
Panstrongylus megistus	45	0	[44]
Cimex lectularius	1,969	102	[45]
Oncopeltus fasciatus	1,115	37	[46]

enzyme that is ubiquitous in nature [26, 91, 92]. That for *T. infestans*, though, was found to belong to a completely different family, that of the 5′-nucleotidase family of enzymes [12]. Interestingly, sand flies [93] express salivary apyrases of the *Cimex* type, while mosquito salivary apyrases belong to the 5′-nucleotidase family [87, 94], clear examples of convergent evolution.

Nudix hydrolases or bis(5′-nucleosidyl)-tetraphosphatases (EC: 3.6.1.17) are enzymes that hydrolyze nucleotides joined by their phosphate groups such as AP4A or AP5A in the case of diadenosine nucleotides, which are known agonists of platelet aggregation and inflammation [95–98]. *C. lectularius* sialotranscriptomes presents clear evidence of such enzymes, but the activity in salivary homogenates was never studied.

Lacking in these Heteroptera sialotranscriptomes are additional nucleotide-acting enzymes, such as endonucleases, found in mosquitoes and sand flies [99–101], and adenosine deaminase, found also in mosquito and some, but not all, sand flies [102–104].

Table 3: Classification of the protein families relevant to secreted products in the Hemiptera/Heteroptera.

Classification	No. of proteins	Genera found[1]	Function characterized?[2]	References
Ubiquitous protein families				
Enzymes				
Cimex apyrase	1	C, R(?)	Y	[26, 50]
5′-nucleotidase	6	T	Y	[12, 13, 51]
Cimex NUDIX hydrolase	3	C		
Secreted esterase	5	C, T		
Inositol phosphate phosphatases including *Cimex* nitrophorins	24	C, T, R	Y/N	[8, 27, 28]
Serine proteases	17	C, T	Y/N	[30]
Chitinase	1	O		
Other enzymes	4	T, O		
Protease inhibitor domains				
Kazal domain containing proteins	13	T		
Serpin	1	C		
Pacifastin-related peptide	1	O		
Cystatin	5	O		
Lipocalins	331	T, R	Y/N	[2, 4–7, 11, 14, 15, 19–21, 23, 43, 52–55]
Salivary OBP	19	C, T, R		
Salivary antigen 5 family	22	C, T, R		
Triatoma dimidiata lectin	2	T		
Immunity related				
Lysozyme	4	C		
Defensin	1	T		
Histidine-rich peptide	1	T		
Immune-related conserved insect protein	1	T		
Arthropod or insect specific families				
Cuticle-like proteins and conserved mucins	5	T		
Conserved insect secreted protein family	6	C, T, O		
Mys2 family	3	R, T		
Cimex-Triatoma family	3	C, T		
Other individual proteins of conserved insect families	4	T		
Hemiptera specific families				
Mys3/hemolysin-like family	16	T, R, O		
Triatoma-specific families				
Trialysin	8	T	Y	[17, 18]
Short trialysin	6	T		
Triatoma matogrossensis family	2	T		
Triatoma matogrossensis family 2	2	T		
Orphan *Triatoma* proteins	19	T		
Rhodnius-specific families				
Orphan *Rhodnius* proteins, include low-complexity proteins	3	R		
Cimex-specific proteins				
Cimex mucin family	2	C		
Orphan *Cimex* proteins	1	C		

Table 3: Continued.

Classification	No. of proteins	Genera found[1]	Function characterized?[2]	References
Oncopeltus-specific families				
Oncopeltus family	3	O		
Oncopeltus family 2	2	O		
Orphan *Oncopeltus* protein	12	O		
Total	559			

[1] C: *Cimex*; T: *Triatoma*/*Dipetalogaster*/*Panstrongylus*; R: *Rhodnius*; O: *Oncopeltus*.
[2] Y: yes; Y/N: characterization of a few or a single member of the family.

3.1.2. Acetylcholinesterases. Four well-expressed and closely related isoforms of a typical acetylcholinesterase enzyme were found in the sialotranscriptome of *C. lectularius* [45]. A single transcript from the same family was also found in *Triatoma matogrossensis*. Although most acetylcholinesterases are extracellular membrane-bound enzymes by virtue of a glycophosphatidyl-inositol membrane anchor in their carboxy termini, these Cimicomorpha enzymes lack this terminal region, and thus these enzymes are secreted. The role of these enzymes in blood feeding is not yet apparent.

3.1.3. Inositol Triphosphate Phosphatases (IPPase) Including Cimex Nitrophorin. This family of proteins has been found ubiquitously in the sialomes of bloodsucking Cimicomorpha, including the well-characterized enzyme from *R. prolixus* [8] and the *C. lectularius* nitrophorin [27–29], a protein found associated with a heme moiety and a carrier and stabilizer of nitric oxide (NO), a very reactive gaseous substance that is also a potent vasodilator and platelet aggregation inhibitor. While the function of *Cimex* nitrophorin is without question, the function of an extracellular inositol phosphatase is puzzling, because these inositol phosphates are intracellular and not available to an extracellular enzyme. Indeed, it appears fitting that inositol polyphosphates should be hydrolyzed, because they perform a proplatelet aggregation function as well as proinflammatory and immune-enhancing roles in leukocytes [105, 106]. Perhaps the enzyme may reach the intracellular pool by some not yet understood mechanism. On the other hand, association of heme with inositol phosphatases seen in the case of *Cimex* nitrophorins is not at all common, being unique to these proteins; investigation of the amino acids that are associated with heme binding does not reveal similarities to other IPPases from either vertebrates or invertebrates (Ribeiro, unpublished).

The phylogram of the IPPase sequences found in Additional File 1 (Figure 1) shows the *Cimex* nitrophorins contained within a strong clade with 100% bootstrap support and constituted by at least three subclades representing at least three genes expressing these NO transporters, plus alleles or other genes. *Cimex* has two additional sequences outside the nitrophorin clade and near the IPPase clade of the remaining triatomines. It is thus interesting that both *Cimex* and triatomines have a common IPPase in their sialome, even though we have no idea of their function. IPPases have not been found in any other transcriptome so far done, including those of mosquitoes, sand flies, biting midges, black flies, and ticks, being thus uniquely from Cimicomorpha blood feeders.

3.1.4. Serine Proteases. Serine proteases are commonly found in the sialotranscriptomes of insects and ticks, as well as in those of Heteroptera. An unusual serine protease activity in the saliva of *T. infestans* has been noted before, but only a partial enzyme purification of the enzyme, named triapsin, was achieved [107]. Within the bloodsucking Heteroptera, only one *Panstrongylus megistus* sequence has been molecularly characterized as a fibrinolytic enzyme [30]. Additional File 1 shows such proteins from Cimicomorpha, including plant-feeding bugs such as *Lygus lineolaris, Lygus hesperus,* and *Creontiades dilutus* [108–110]. The phylogram of these enzymes (Figure 2) shows two well-defined clades, one containing most of the *Lygus* sequences, but also two *T. matogrossensis* and one *T. brasiliensis* sequence, within a clade of 86% bootstrap support, suggesting a common ancestral salivary serine protease for plant- and blood-feeding Cimicomorpha. The fibrinolytic enzyme of *Panstrongylus* shares a strongly supported clade with two other *T. matogrossensis* sequences, which are probable orthologs of the *Panstrongylus* gene. The *Cimex* sequence appears as an outlier to the group. *Rhodnius* sialotranscriptomes have not revealed proteases, and its saliva does not hydrolyze the substrates used in the characterization of the *T. infestans* triapsin (Ribeiro, unpublished).

3.1.5. Other Enzymes. A chitinase and a lipase were found in *Oncopeltus*, while *T. matogrossensis* displayed a salivary phospholipase and a metalloprotease. The precise role of these enzymes is unknown. Salivary metalloproteases in ticks have been associated with fibrinolytic and antiangiogenic activities [111, 112], while the *Oncopeltus* enzyme may be associated with digestive or antifungal functions.

3.2. Protease Inhibitor Domains

3.2.1. Kazal Domain-Containing Peptides. The Kazal domain occurs in many protease inhibitors, and its structure was first determined for the proteinase inhibitor IIA from bull seminal plasma [113]. The sialotranscriptome of members of South American *Triatoma* (*T. infestans, T. matogrossensis,* and *T. brasiliensis*) but not North American *T. dimidiata* or *T. rubida,* nor any other sialotranscriptome of Cimicomorpha, abounds with transcripts coding for proteins containing this

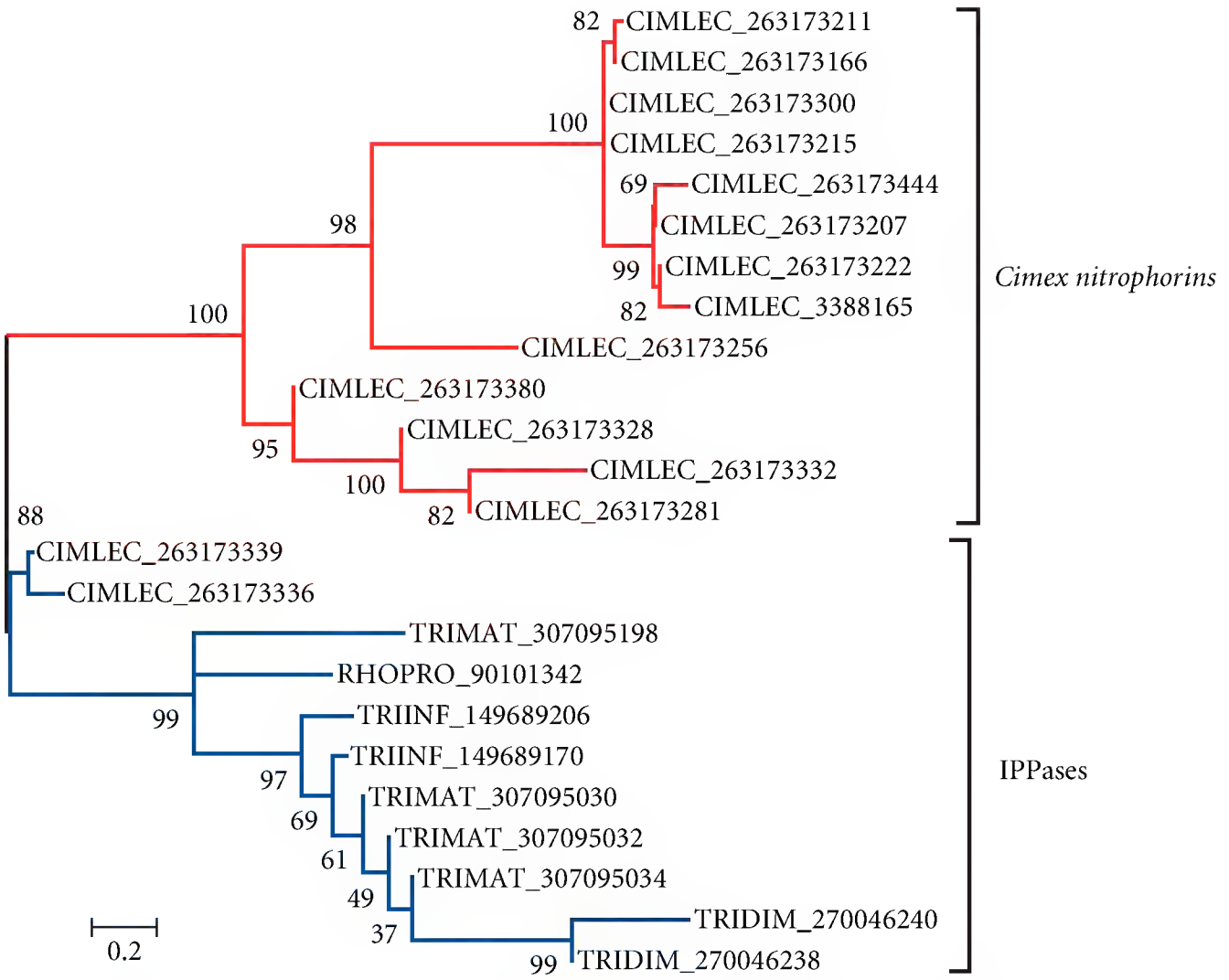


Figure 1: Phylogram of the inositol triphosphate phosphatase family of Cimicomorpha. The sequences are named with the first 3 letters of the genus name, followed by the first 3 letters of the species name, followed by their GenBank GI accession number. The sequences were aligned by Clustal, and the neighbor-joining bootstrapped phylogram was obtained with the MEGA package with 10,000 iterations, Poisson model of amino acid substitution and pairwise amino acid comparisons using the gamma rate of amino acid substitution (gamma parameter = 1). The numbers at the nodes are the percent bootstrap support. The line at the base indicates the rate of amino acid substitution per site.

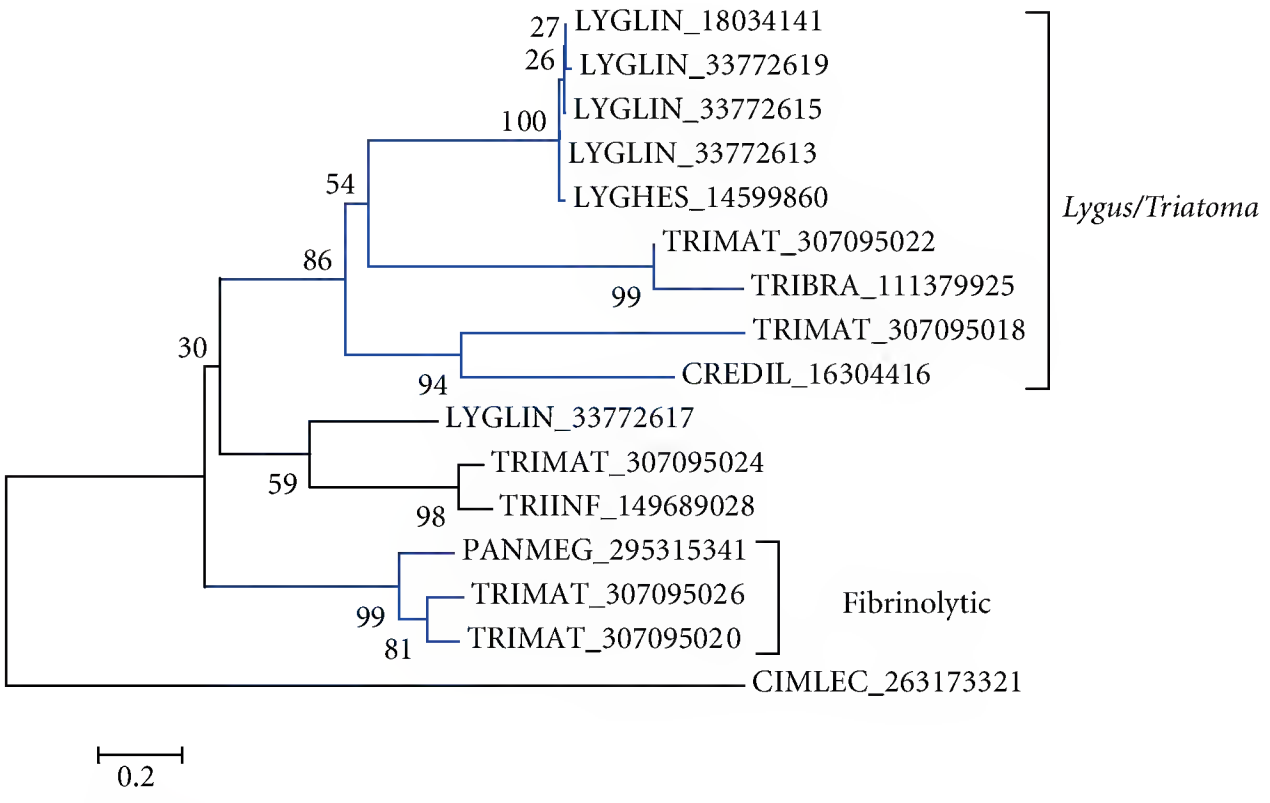


Figure 2: Phylogram of the salivary serine proteases of Cimicomorpha. See the legend of Figure 1 for more details.

domain; however, none have been so far characterized functionally. In *Rhodnius, Triatoma,* and *Dipetalogaster*, the crop antithrombin has been characterized as a protein containing two such domains [114–117], but salivary anticlotting of *Rhodnius* and *Triatoma* has been shown to be different lipocalins named prolixin S and triabin [2, 21, 118]. Kazal-type peptides can function as antimicrobials by inhibiting microbial exoproteases essential for their survival [119, 120] and can also work as vasodilators, as in the case of a tabanid salivary protein named vasotab, which is suspected to modify

ion channels [121]. These functions should be taken into consideration in functional assays of the recombinant Kazal peptides.

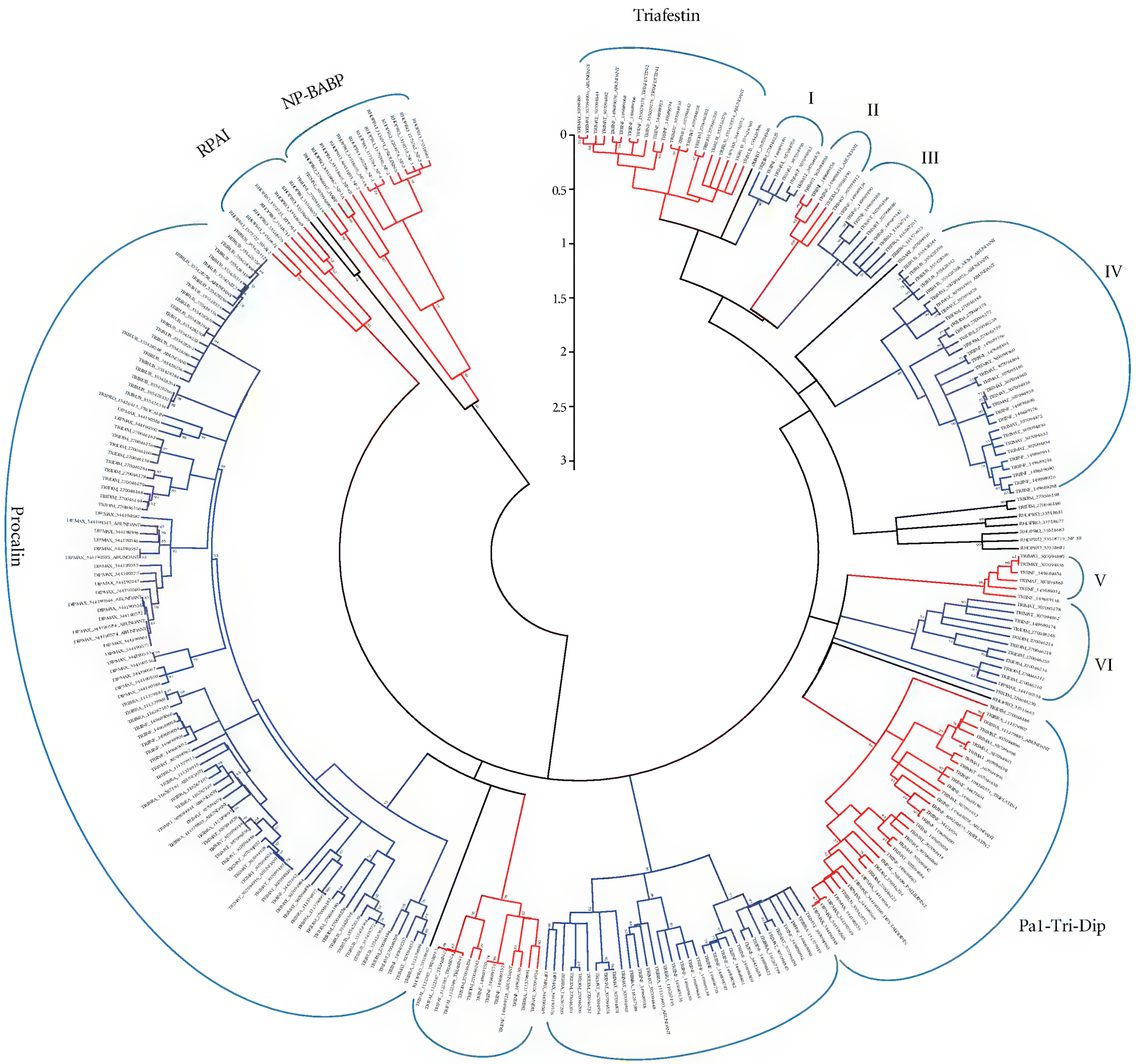
3.2.2. Serpin. The serine protease inhibitor (serpin) family is ubiquitous in nature, functioning mostly as endogenous regulators of proteolytic cascades such as inhibiting thrombin in vertebrates (plasmatic antithrombin 3) or regulating phenol oxidase activation cascades in invertebrates [122, 123]. The salivary anticlotting proteins of *Aedes* mosquitoes (but not those of anopheline mosquitoes) are members of this family [124, 125]. A single sequence of this family, derived from four ESTs, was found in the sialotranscriptome of *C. lectularius*. Its target is still unknown.

3.2.3. Pacifastin and Cystatin. Proteins containing these domains were only found in the sialotranscriptome of *Oncopeltus*. Pacifastins are typical serine protease inhibitors of insects and crustaceans [126], while cystatins are ubiquitous proteins typically inhibiting cysteine proteases [127]. Although a single EST was found coding for the pacifastin peptide, five well-expressed cystatins were identified in *Oncopeltus*. The targets of these peptides are unknown, but it was suggested that the salivary cystatins may prevent plant apoptosis induced by cysteine proteases [46, 128, 129]. Tick sialomes have revealed cystatins that were shown to inhibit inflammation and maturation of dendritic cells in their hosts [130].

3.3. Lipocalins. The term lipocalin literally means a cup of lipid, as these proteins form a barrel with a hydrophobic interior cavity that is suitable to transport lipids and other hydrophobic compounds in an aqueous milieu [131–133]. There is virtually no sequence conservation in the family, which is recognized by its typical 3D structure composed of a repeated +1 topology β-barrel. This protein family is by far the most abundant in sialotranscriptomes of triatomine bugs (see review [132]) but remarkably absent in *Cimex* and *Oncopeltus*; however, it was also abundantly recruited in tick sialomes [56], another case of convergent evolution. Additional File 1 provides for 331 lipocalins, which is more than half of all putative secreted proteins listed in this work. Several of these proteins may be alleles of the same gene. The sheer size of the family in individual species is indicative of gene duplication events that might have had an impact during the evolution of blood-feeding [134–136]. Following gene duplication—by retrotransposition or more commonly by forming tandem repeats due to transposable element recombination—the new genes can lead to an increased transcript load in a particular organ or tissue. If this augmented expression increases fitness (e.g., helps the bug to feed), the gene will persist; otherwise, it will evolve to be a pseudogene [137]. Once genes are duplicated and fitness is increased by the duplication, these are free to evolve independently and to diverge from each other by acquisition of novel functions. Salivary genes of bloodsucking arthropods are under selection by two different processes. First, the gene can evolve in the direction of fine tuning its function in relationship to its target. For example, a bug feeding on a bird may have "ideal" anticlotting, but if ecologic changes appear and the bug shifts to another host, this anticlotting may still work but have some room for improvement (e.g., by increasing its affinity to the specific thrombin). Second, any protein injected into the skin of a vertebrate is capable of eliciting an immune reaction, which may lead to defensive host behavior following mast cell degranulation or complement-mediated local inflammation, leading to interruption of the meal or killing of the insect. This may lead to a scenario of balanced polymorphism, with the least common epitope being the best one to have, thus multiplying the number of different alleles in a population that are selected to have the same optimal function but the least common antigenicity. Host immune pressure can also lead to gene obsolescence, creating a niche for cooption (exaptation) of new genes, including horizontal transfer [138], which may substitute for the lost function and thus may explain the appearance of novel salivary genes in related organisms [139].

Lipocalin functions in triatomines are multiple and linked to their unique barrel when working as kratagonists (from the Greek kratos = seize) [140], which are binders of relatively small agonists such as biogenic amines, TXA_2, leukotrienes, or ADP, or carrying the heme that carries NO in *Rhodnius* nitrophorins, or functions linked to their side chains when they work as anticlotting agents such as triabin (for references for these functions, see Table 1). Uniquely, the protein nitrophorin 2 from *R. prolixus* has three functions: (i) it carries NO, (ii) it binds histamine, and (iii) it is an inhibitor of the activation of Factor X [5, 141]. Notice that contrary to their names as "lipid cups," many of these lipocalin ligands are well charged and not hydrophobic, such as biogenic amines and ADP. The functions of the salivary lipocalins in ticks are similarly associated with their kratagonist activity toward biogenic amines or arachidonic acid derivatives, or as inhibitors of complement activation [142–148].

A phylogram of the triatomine lipocalins, although a bit overwhelming in size, presents a bird's-eye view of the several distinct families arranged mostly in robust clades (Figure 3 and Additional File 2). Most clades have not a single member that has been analyzed functionally (marked with Roman numerals in Figure 3), including the clade containing the *Triatoma protracta* antigen procalin; accordingly there are eight clades that have no known function. Additional File 2 is provided for high-resolution display of the sequences, which have their NCBI accession numbers for sequence retrieval. A few details deserve some comments with respect to the phylogram. (i) The clade named Pal-Tri-Dip contains the *Triatoma* proteins pallidipin, triplatin, and the *Dipetalogaster* protein dipetalodipin, which are platelet inhibitors possibly all due to being TXA_2 kratagonists as demonstrated for triplatin and dipetalodipin [25], thus indicating the conservation of this function among two different genera. (ii) Most *Rhodnius* lipocalins cluster in two clades, one containing all the known NO carriers, named nitrophorins (NP) and the other containing the adenosine nucleotide kratagonists named RPAI (*Rhodnius* platelet aggregation inhibitor). (iii) The *Rhodnius* biogenic amine-binding protein (BABP)



Triabin VII

FIGURE 3: Phylogram of the salivary lipocalin family of triatomines. Clades containing members that have been functionally characterized are named according to these proteins. Other clades are named with Roman numerals. Except for the triafestin clade, other clades have >70% bootstrap support. The sequences are named with the first 3 letters of the genus name, followed by the first 3 letters of the species name, followed by their GenBank gi| accession number. When the protein has been functionally characterized, its name is also included after the NCBI number. Abundantly expressed proteins are also marked to indicate this fact. The numbers in the bar indicate the rate of amino acid substitution per site. For other details, see Figure 1 legend.

somewhat surprisingly clusters with the nitrophorins, but BABP does not have a heme group and has higher affinity for serotonin and norepinephrine, constituting a good example of gene duplication and divergence of function [7]. (iv) Exceptionally, one *T. matogrossensis* and one *T. dimidiata* protein sequence group with the NP-BABP clade with 99% bootstrap support. The function of these proteins could lead to the original function of the *Rhodnius* nitrophorins, which are exclusive of the genus *Rhodnius*. Indeed the abundance of these heme proteins in *Rhodnius* salivary glands makes these glands distinctively bright cherry red in color, as first pointed out by Wigglesworth nearly 70 years ago [149]. *Triatoma* and *Dipetalogaster* glands are clear or of a very pale yellow color [150]. (v) *Rhodnius* lipocalins not belonging to the NP-BABP and RPAI clades are scattered in the phylogram, including one sequence between the procalin and triabin

clades, one between the triabin and VI clades, and a group of four proteins between clades IV and V. None of these *Rhodnius* proteins group within strong bootstrap support to any of the *Dipetalogaster*- or *Triatoma*-containing clades. (vi) Finally, the procalin clade is very extensive and contains many robust subclades, many of which are of single species, indicating possible recent events of gene duplication or extensive polymorphism.

3.4. Odorant/Pheromone-Binding Family (OBP). The OBP family, like the lipocalins, is specialized in carrying small hydrophobic ligands in aqueous media [151, 152]. A modified version of the odorant-binding family of proteins is very abundant in the sialotranscriptomes of hematophagous Nematocera [138] and named as the D7 protein family. A few mosquito proteins have been crystallized and functionally characterized, showing kratagonist activity toward biogenic amines, TXA_2, and leukotrienes, in addition to anticlotting activity [153–156].

In hematophagous Cimicomorpha, members of the OBP family are found in *Rhodnius, Triatoma,* and *Cimex* but are particularly abundant in *Cimex*, with two OBP proteins having over 250 ESTs in a total of ~2,000 ESTs, suggesting the OBP family has been recruited by *Cimex* to function as the lipocalins in triatomines. No salivary member of this family in Cimicomorpha has been so far functionally characterized.

3.5. Antigen-5 Family. This is a ubiquitous protein family found in plants and animals, including expression in the venom glands of vespids, where it was recognized as an antigen, thus the name antigen 5 for this family. They are members of the CAP superfamily, most with unknown function [157]. In snakes and lizards, they have been associated with venom toxins [158–160]. In stable flies, one salivary antigen 5 protein binds immunoglobulins and may function as an inhibitor of the classical pathway of complement activation [161]. In horse flies, one protein has acquired a disintegrin motif and is a strong inhibitor of platelet aggregation [162–164]. All triatomine sialotranscriptomes have revealed this class of proteins, which is particularly abundant in *Dipetalogaster.* The function of these proteins in triatomine blood feeding is still unknown.

3.6. Lectin. Triatoma dimidiata exclusively presents two partial sequences containing a galactose-binding domain. While lectins—mainly C-type lectins—have been described in the sialotranscriptome of mosquitoes (none with known function), this is so far a unique finding in triatomine sialotranscriptomes.

3.7. Immunity-Related, Ubiquitous Families. Immunity-related proteins and peptides are commonly found in the saliva of bloodsucking arthropods and may help to control microbial growth in the ingested meal and perhaps also avoid microbial infection of the bite site. Lysozyme, while common in mosquito sialomes, is found exclusively so far in *Cimex* sialomes, with four quite different proteins being reported. *D. maxima* presents a histidine-rich peptide that could function as an antimicrobial peptide, and a defensin is reported from *T. infestans.* The absence of commonly found salivary antimicrobial peptides in triatomines suggests that if this salivary function is present within these organisms, it may be encoded by lineage-specific gene families, one of which (trialysin) will be reported further below.

4. Arthropod-Specific Families

Several insect-specific families are further identified, none functionally characterized, and most without domains providing a clue for their function. These include proteins with chitin-binding domains and cuticle-like homologs, which may be associated with salivary ducts rather than a function in the injected saliva. One conserved secreted insect protein family of basic peptides having ~100 amino acids after signal peptide cleavage occurs in *Cimex, Oncopeltus,* and *Triatoma* sialotranscriptomes. Homologs are found by blastp to the nonredundant (NR) protein database including a venom protein from the wasp parasitoid *Nasonia vitripennis* identified in a proteomic study [165]. Exceptionally, there are also homologs to proteins from the soil bacteria *Streptomyces clavuligerus,* having 52% identity to the insect proteins. Similarly, the protein originally described in *R. prolixus* as MYS2 has homologs found in the sialotranscriptomes of *T. brasiliensis* and *T. matogrossensis* and is similar to many other insect proteins in the NR, including protein sequences deducted from the sialotranscriptome of the tsetse *Glossina morsitans* [166]. Three sequences, one each from *C. lectularius, T. infestans,* and *T. matogrossensis,* have 25% amino acid sequence identity but 52% similarity and little similarity to other proteins on the NR database. These sequences are grouped in Additional File 1 as the *Cimex-Triatoma* family. PSI-blast initiated by the *T. matogrossensis* sequence against the NR database initially retrieves only the two other sequences, but on first iteration it retrieves dozens of insect proteins (Additional File 3), and in the third iteration it retrieves *Daphnia* and tick proteins, suggesting this is an arthropod family of high divergence. Finally, the sialotranscriptome of *T. matogrossensis* identified four additional nonrelated proteins that have insect homologs but were not found in other reported sialotranscriptomes of Hemiptera but are similar to proteins reported from *G. morsitans* and from *Aedes aegypti* sialotranscriptomes. It is possible that these families function as antimicrobial peptides, but so far none has been characterized.

5. Hemiptera-Specific Families

5.1. Mys3/Hemolysin Family. When the *R. prolixus* sialotranscriptome was reported [36], an additional mysterious protein was named Mys3. Later, with additional sialotranscriptome reports, another protein family emerged, named as hemolysin-like because some members had weak similarity to bacterial proteins annotated as hemolysins. PSI-blast later revealed that these proteins all belong to a single family that is quite divergent, including a truncated protein from the sialome of *Oncopeltus*, suggesting a non-blood-feeding role, perhaps antimicrobial, for its members.

5.2. Triatoma-Specific Families. Sialotranscriptomes of several species of the *Triatoma* genus reveal several unique protein families, among which are the trialysin and short trialysin families. The trialysins are basic proteins of mature MW near 26 kDa that can be further processed to peptides that have lytic properties [17, 18] and may function as antimicrobials. Short trialysins have mature MW of ~6.1 and acidic pI and are so named because they match the amino terminal region of the mature trialysins. Both forms are abundantly expressed but only found in *T. infestans* and *T. matogrossensis,* which are from southern South America, and are not found in the sialotranscriptomes of *T. brasiliensis,* found in northeastern Brazil, or on those of the North American *T. dimidiata* or *T. rubida*. Additional File 1 reports 19 protein sequences from *Triatoma* that are not similar to anything deposited in the NR database and two pairs of sequences from *T. matogrossensis* that only match its pair members. None has been functionally characterized. It is interesting that of these 23 sequences only one derives from *T. rubida* and the remaining derive from *T. infestans* and *T. matogrossensis*, although the number of clones sequenced for the *T. brasiliensis*, *T. dimidiata,* and *T. rubida* was similar to those of *T. infestans* and *T. matogrossensis*, suggesting a greater sialome diversity in these bugs from southern South America.

5.3. Rhodnius-, Cimex-, and Oncopeltus-Specific Families. Additional File 1 presents 16 proteins from the bugs named above that have no significant matches to the NR database except in some cases for some proteins of low complexity. None of these proteins has been functionally characterized. This includes *Rhodnius* MY1 protein, one of three mysterious proteins revealed in the first bug sialotranscriptome [36]. As seen above, MYS2 and MYS3 were later found to be members of larger families. It is expected that, with a larger number of genomes and transcriptomes sequenced, MYS1—as well as the other orphan proteins in this group—will also be deorphanized.

6. Housekeeping Proteins

Mostly from the sialotranscriptomes shown in Table 2, many housekeeping protein sequences were also deduced, including many associated with energy metabolism, protein synthesis, modification, and export, among other classes (see worksheet named "Housekeeping" of Additional File 1). Interestingly, the sialotranscriptome of *Triatoma rubida* shows abundant expression of members of the cytochrome P450 as well as of the 15-hydroxyprostaglandin dehydrogenase, suggesting either that the salivary gland may have an active endogenous prostaglandin signaling or that prostaglandins may be secreted in the saliva of these bugs. Cyt P450 transcripts were also detected in *Rhodnius* and *T. matogrossensis,* and the prostaglandin dehydrogenase was also found in *T. infestans.* Increased depth of sequencing of these sialotranscriptomes may certainly reveal these two classes of proteins to be expressed in all triatomines.

7. Concluding Remarks

Blood-feeding Cimicomorpha have developed a sophisticated and divergent array of salivary pharmacologically active compounds that disarm their hosts' reaction against blood loss. In a few transcriptomes encompassing members of the Reduviidae and Cimicidae, the convergent evolution scenario in the sialomes of these two families is apparent. Both have apyrase activity, but from different gene families; *Cimex* and *Rhodnius* (but not any Triatomini member) use NO as a vasodilator but co-opted completely different heme proteins to carry this unstable gas. The anticlotting compounds are different at the Reduviidae tribe level and so on. The lipocalin expansion is remarkable among the triatomines and nonexistent in *Cimex.* These proteins can play many different functions as binders of small agonists (kratagonists), NO carriers, or protease inhibitors. In *Cimex*, the expanded odorant binding family may have taken this role, but none thus far has been characterized.

Notice that the sialome of *Oncopeltus*, a member of the Pentatomomorpha—the most closely related suborder to the Cimicomorpha (see http://tolweb.org/Heteroptera/10805) [76, 167]—revealed virtually nothing in common with the Cimicomorpha, and the Cimicidae sialome also revealed little in common with the Reduviidae, perhaps as expected by the divergence of these families (see http://tolweb.org/Cimicomorpha/10817). Zooming-in on the Triatomine group, it will be interesting in the future to describe the sialomes of additional tribes of the Triatomine, such as the Bolboderini, which includes bugs that feed on insect hemolymph, the Cavernicolini that are associated with bats, and members of the Linshcosteus genus that are found in India [168] and could be divergent members. Zooming a little out and as indicated by Schofield and Galvão [49], facultative blood feeding is found in non-Triatominae members of the Reduviidae, including the Emesinae, Harpactorinae, Peiratinae, Physoderinae, and Reduviinae. Sialomes of these subfamilies could be more indicative of the prevalent "pre-adaptations" available as stepping stones and promoted by the blood-feeding habit. On the other hand, the *Cimicidae* are closely related to the bat bugs (Polyctenidae), which is a sister group, and to the Anthocoridae (flower bugs; http://tolweb.org/Cimicomorpha/10817), which feed on small insects. These non-blood-feeding closer relatives may reveal insights into the Cimicidae evolution to hematophagy.

Acknowledgments

This work was supported by the Intramural Research Program of the Division of Intramural Research, the National Institute of Allergy and Infectious Diseases, and the National Institutes of Health. The authors thank NIAID DIR intramural editor Brenda Rae Marshall for editing the manuscript. Because JMCR and IMBF are government employees and this is a government work, the work is in the public domain in the United States. Notwithstanding any other agreements, the NIH reserves the right to provide the work to PubMedCentral for display and use by the public, and

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 150958, 5 pages
doi:10.1155/2012/150958

Research Article

Effect of Crude Plant Extracts on Mushroom Mite, *Luciaphorus* sp. (Acari: Pygmephoridae)

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Received 12 April 2012; Accepted 20 May 2012

Academic Editor: Kabkaew Sukontason

The use of plant extracts for controlling agricultural pests has become increasingly popular in the recent years. Mushroom mite, *Luciaphorus* sp., is a destructive pest of several mushroom species and has been reported to cause severe loss of mushroom cultivation in many settings. The efficacies of 23 rhizome and leaf extracts were evaluated against female adults of *Luciaphorus* sp. At 3 days after treatment, the rhizome extracts derived from *Curcuma xanthorrhiza* Roxb. and *Zingiber montanum* (Koenig) Link ex Dietr. were found to have very strong acaricidal activities, resulting in 100% mite mortality, followed by *Curcuma longa* Linn. (98.89%), *Zingiber zerumbet* (L.) Smith. (97.78%), *Kaempferia parviflora* Wall. Ex Baker (88.89%), and *Zingiber officinale* Roscoe. (84.44%). The leaf extracts of *Ocimum sanctum* Linn. and *Melissa officinalis* L. also resulted in 100% mite mortality 3 days after treatment, while the other leaf extracts induced mite mortality only below 70%. The results suggested that rhizome extracts of *C. xanthorrhiza* and *Z. montanum* and leaf extracts of *O. sanctum* and *M. officinalis* have a great potential for future development as natural acaricides for controlling *Luciaphorus* sp.

1. Introduction

Luciaphorus sp. (Acari: Pygmephoridae) is considered as one of the most destructive pests of mushroom cultivation in Thailand. This pygmephorid mite is responsible for the severe production losses of *Lentinus squarrosulus* (Mont.) Singer, *L. polychrous* Lev., *Auricularia auricula-judae* (Bull.:Fr.) Wettst. and *Flammulina velutipes* (Curt.:Fr.) Karst. mushrooms in the Northeast of Thailand [1]. Despite that, little is known about the effective measures for controlling this mite and routine horticultural hygiene is the only procedure to alleviate the problem. To make the situation worse, desperate mushroom growers in Thailand use a large amount of carbamate and organophosphate insecticides and even some harmful solvents to manage this mite; however, this results in very limited success [2].

As a consequence, this mite becomes rapidly resistant and more harmful miticides have to be applied. The use of toxic miticides raises the concerns because of their effects on environments, human safety, and nontarget organisms. Hence, the use of nontoxic natural products for controlling this agricultural pest has been proposed. There are several higher plants that are rich in natural substances, especially the secondary metabolites, such as terpenes, steroids, alkaloids, phenolics, and cardiac glycosides, and can be used as nonharmful, environmentally friendly agents for insect control. Indeed, the use of natural compounds derived from plant extracts has been suggested as alternative treatments for insect and mite controls due to their multiple modes of action, including repellence, feeding and oviposition deterrence, toxicity, and growth regulatory activity [3–6]. Moreover, plant-based pesticides are often found to contain a mixture of active substances which can delay or prevent resistance development [7]. Therefore, in this study, the acaricidal activities of 23 plant extracts were determined against the mushroom mite, *Luciaphorus* sp.

2. Materials and Methods

2.1. Mushroom and Mite Culture. Lentinus squarrosulus Mont. mushroom culture was obtained from the Mushroom Growers Society of Thailand. The mycelium was freshly sub cultured on 90 mm plastic Petri dish plates containing potato dextrose agar (PDA, Sigma) and grown at 25°C.

Luciaphorus sp. mites were collected from infested *L. squarrosulus* composts obtained from Rapeephan mushroom farm in Khon Kaen province in the Northeast of Thailand. A pair of male and female mites was maintained at 28°C using fresh *L. squarrosulus* spawn that was grown on sawdust and sorghum grains in a glass bottle. The offspring that were in-house bred inside this glass bottle were used for all the experiments.

2.2. Preparation of Plant Extracts. Leaves and rhizomes of 23 plants were collected locally from Mahasarakham province in the Northeast of Thailand (Table 1). Plant materials were cut into small pieces and dried in hot air oven at 45°C for 3 days.

The dried plants were separately ground into powders using a small grinder and stored at 4°C in polypropylene bags. For extraction, 100 g of each powdered plant materials and 300 mL of 80% ethyl alcohol were added into sterile 2L Erlenmeyer flask, and the flask was agitated at 100 rpm for 24 h. After filtering through a Buchner funnel and Whatman No. 1 filter paper, the extracts were concentrated under low pressure using rotary evaporator. The crude extracts were reconstituted to have the concentration of 20% (w/v) using 80% ethyl alcohol (v/v, in distilled water) and stored at 4°C in glass vials to be used as stock plant extracts. For the tests, these stock plant extracts were dissolved in distilled water containing 0.05% Tween 80 to have the concentration of 5% (w/v).

2.3. Bioassay. For evaluation of each plant extract, 100 adult female mites were transferred to a 50 mm Petri dish plate containing mushroom mycelial culture grown on PDA medium, and the plate was then sprayed with 500 μL of each plant extracts prepared at the concentration of 5% (w/v). The same volumes of the sterilized distilled water (DW) and 0.005% Omite (commercial miticide) were used as control groups. The experiments for each plant extracts were performed in triplicates. All plates were incubated in the growth chamber at 28°C and 85% RH in the dark. The mortality of mites was recorded every day for 5 days after application with plant extracts.

2.4. Statistical Analysis. Data on the percentages of mite mortality due to application with plant extracts were arcsine-transformed and subjected to analysis of variance using the general linear models procedure (SAS Institute, Cary, NC, USA). Significant differences between the treatments were determined using the LSD test at $P < 0.05$.

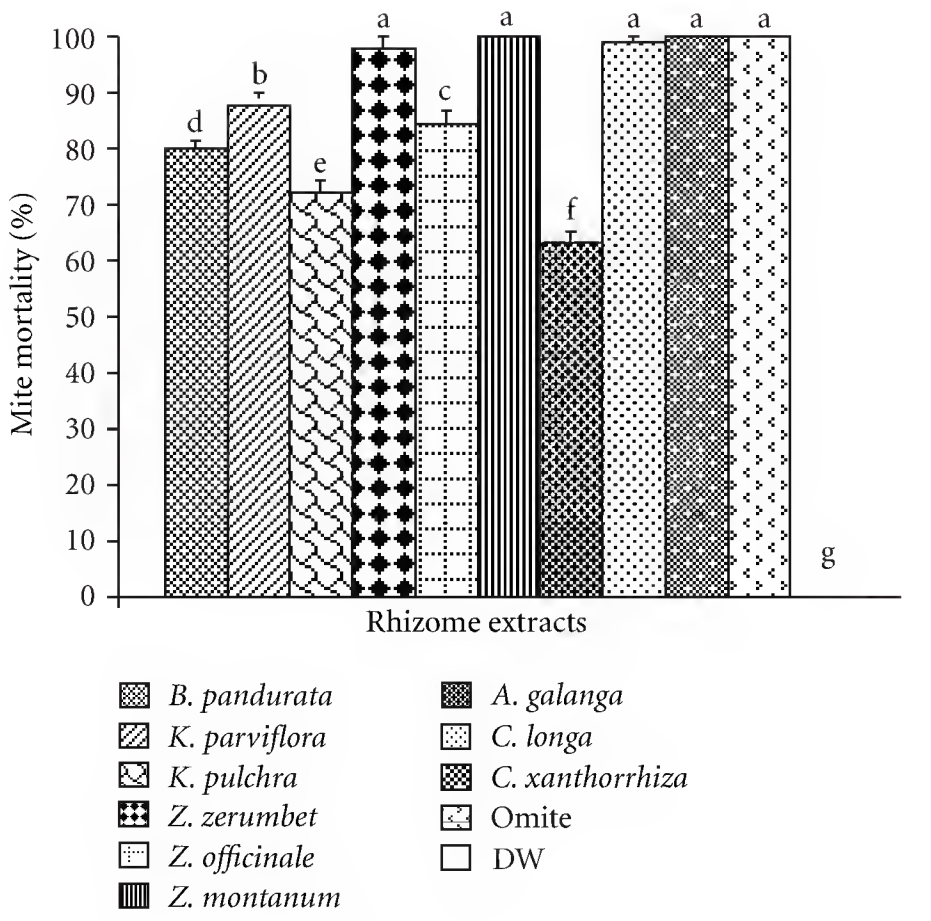

FIGURE 1: The mortality rates of adult female *Luciaphorus* sp. after being treated with 5% rhizome extracts at 3 days after application. Bars (mean ± SE) with same letter(s) are not significantly different as determined by LSD test at $P < 0.05$.

3. Results

3.1. Acaricidal Activities of Rhizome Extracts. In this study, all rhizome extracts were shown to have acaricidal activities against *Luciaphorus* sp., and the percentages of mite mortality increased progressively and reached the plateau after 3 days of applications (Figure 1). On day 3, the significantly high levels of mortality rates were caused by the rhizome extracts of *C. xanthorrhiza* (100%), *Z. montanum* (100%), *C. longa* (98.89%), and *Z. zerumbet* (97.78%), followed by *K. parviflora* (88.89%), *Z. officinale* (84.44%), *B. pandurata* (80.00%), *K. pulchra* (72.22%), and *A. galanga* (63.33%) (Figure 1). Interestingly, on day 1, *K. parviflora*, *Z. officinale*, *C. longa*, and *C. xanthorrhiza* extracts resulted in mortality rates at over 70% which were significantly higher than the other treatments (data not shown). However, on day 2, mite mortality rates in almost all treatments were over 70% with the exception of *A. galanga* (56.67%) and *K. pulchra* (67.78%) (data not shown).

3.2. Acaricidal Activity of Leaf Extracts. The levels of mite mortality after applications with leaf extracts also reached maximum on day 3 (Figure 2). On day 3, the leaf extracts of *O. sanctum* and *M. officinalis* resulted in maximum mortality (100%), but the other treatments were shown to result in mortality at levels below 70% (Figure 2). This was not unexpected because only the applications with the leaf extracts of *O. sanctum* and *M. officinalis* caused over 70% of mortality on day 1 (data not shown). Also, on day 2, mortality rates in all treatments increased and the leaf extracts of *O. sanctum* and *M. officinalis* still resulted in mite mortality at the levels significantly higher than the rest,

Table 1: Plants and their parts used for evaluation of acaricidal activities against *Luciaphorus* sp.

Scientific name	Family	Common name	Parts
Boesenbergia pandurata (Roxb) Schltr.	Zingiberaceae	Fingerroot	Rhizome
Kaempferia parviflora Wall. Ex Baker	Zingiberaceae	Belamcanda chinensis	Rhizome
Kaempferia pulchra (Ridl.) Ridl.	Zingiberaceae	Peacock ginger, resurrection lily	Rhizome
Zingiber zerumbet (L.) Smith.	Zingiberaceae	Wild ginger, Martinique ginger	Rhizome
Zingiber officinale Roscoe.	Zingiberaceae	Ginger	Rhizome
Zingiber montanum (Koenig) Link ex Dietr.	Zingiberaceae	Phlai, cassumunar	Rhizome
Alpinia galanga (L.) Swartz.	Zingiberaceae	Kha, galingale, galanga	Rhizome
Curcuma longa Linn.	Zingiberaceae	Turmeric	Rhizome
Curcuma xanthorrhiza Roxb.	Zingiberaceae	Curcuma	Rhizome
Cymbopogon citratus Stapf.	Gramineae	Takhrai, lemongrass	Leaf
Citrus hystrix DC.	Rutaceae	Leech lime	Leaf
Ocimum basilicum Linn.	Labiatae	Ho-ra-pa, sweet-basil, common basil	Leaf
Ocimum canum Linn.	Labiatae	Hairy basil	Leaf
Ocimum sanctum Linn.	Malvaceae	Holy basil, sacred basil	Leaf
Moringa oleifera Lam.	Moringaceae	Horse radish tree	Leaf
Annona squamosa Linn.	Annonaceae	Sugar apple	Leaf
Psidium guajava Linn.	Myrtaceae	Guava	Leaf
Eucalyptus camaldulensis Dehnh.	Myrtaceae	Red river gum, Murray red gum, red gum	Leaf
Artocarpus heterophyllus Lam.	Moraceae	Jackfruit tree	Leaf
Piper sarmentosum Roxb. Ex Hunter.	Piperaceae	Cha-plu	Leaf
Murraya paniculata (L.) Jack.	Rutaceae	Orange jessamine, satin-wood,	Leaf
Melissa officinalis L.	Lamiaceae	Kitchen mint, marsh mint	Leaf
Cassia siamea (Lam.) Irwin et Barnaby	Fabaceae	Kassod tree, siamese senna, Thai copperpod, siamese cassia	Leaf

accounting for 97.78% and 94.44%, respectively (data not shown).

4. Discussion

Several plants have been found to contain bioactive compounds with a variety of biological actions against insects and mites, including repellent, antifeedant, anti-ovipositional, toxic, chemosterilant, and growth regulatory activities [4, 8]. Therefore, botanical insecticides have long been recommended as attractive alternatives to synthetic chemical insecticides for pest management because these chemicals pose little threat to the environment or to human health [9]. For example, the crude foliar extracts of five subfamilies of Australian Lamiaceae, including Ajugoideae, Scutellarioideae, Chloanthoideae, Viticoideae, and Nepetoideae, were found to have contact toxicity against the polyphagous mite (*Tetranychus urticae* Koch) [10]. This *T. urticae* could also be inhibited by the essential oil in crude foliar extract of sandalwood (*Santalum austrocaledonicum*), resulting in 87.2 ± 2.9% mortality and 89.3% reduction of the total number of eggs on leaf disks treated with this oil [11]. Piperoctadecalidine, which is the alkaloid isolated from *Piper longum* Linn., was also found to have activities against *T. urticae* at LD_{50} of 246 ppm [12]. Moreover, Aslan et al. [13] reported that essential oil vapours from *Satureja hortensis* Linn., *Ocimum basilicum* Linn, and *Thymus vulgaris* Linn. had potential against *T. urticae*, but the essential oil obtained from *S. hortensis* was the most effective at 1.563 μL/L air dose by causing 100% mortality of *T. urticae* after 4 days of treatment.

In recent years, many studies have also been conducted to investigate the activities of plant extracts or essential oils against carmine spider mite (*Tetranychus cinnabarinus* Boisd. Tunc) and Hawthorn red spider mite (*Tetranychus viennensis* Zacher). The chloroform extract of *Kochia scoparia* Linn. was shown to have rapid acaricidal activities against *T. urticae*, *T. cinnabarinus*, and *T. viennensis*, resulting in the highest mortality at 92.58, 88.88, and 84.47%, respectively, within 24 h after treatment [14]. Also, toxicity against *T. cinnabarinus* and *T. viennensis* could be quickly induced by the petroleum ether extract of *Juglans regia* Linn., resulting in mortality rates at 81.58 and 78.58%, respectively, within 24 h [7].

Furthermore, the complete 100% mortality of *T. cinnabarinus* was found to be induced by the essential oils of *Cuminum cyminum* Linn., *Pimpinella anisum* Linn., and *Origanum syriacum* var. *bevaii* (Holmes) as fumigants in greenhouse experiments [15]. This complete mortality could also be produced by using the acetone parallel extract of *Artemisia annua* Linn. leaves collected in July [16]. In addition, Zhang et al. [17] reported that benzene extracts derived from *C. longa* Linn. had LC_{50} against *T. cinnabarinus* at 99.3 ppm after 72 h. The high mortality rates of *T. cinnabarinus* could be induced by methanol extracts of *Gliricidia sepium* (Jacq) Kunth ex Steud. (100%) and *Lippia origanoides* Kunth (96.6%) when used at the concentration of 20% [18]. Additionally, Sertkaya et al. [8] evaluated the

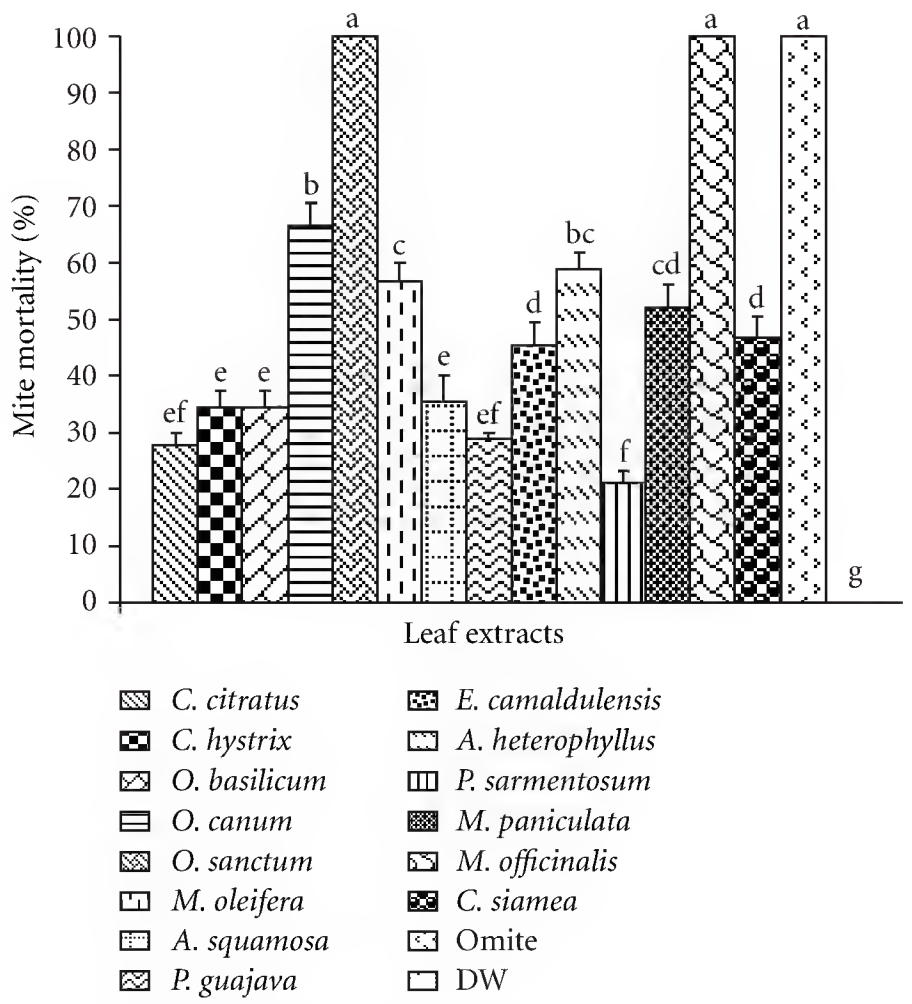

FIGURE 2: The mortality rates of adult female *Luciaphorus* sp. after being treated with 5% leaf extracts at 3 days after application. Bars (mean ± SE) with same letter(s) are not significantly different as determined by LSD test at $P < 0.05$.

efficacy of essential oils derived from medicinal plants against *T. cinnabarinus* and showed that thyme (*Thymbra spicata* Linn. subsp. *spicata*), oregano (*Origanum onites* Linn.), mint (*Mentha spicata* Linn.), and lavender (*Lavandula stoechas* Linn. subsp. *stoechas*) essential oils had LC_{50} values of 0.53, 0.69, 1.83, and 2.92 ppm, respectively. Moreover, the acetone extract of *Aloe vera* Linn. leaves was shown to have acaricidal activity against female *T. cinnabarinus* at 3 days after treatment with LC_{50} value of 90 ppm [6].

Other insect pests were also found to be inhibited by plant extracts. According to the results of Liu et al. [19], the ethanol extracts of *Eupatorium adenophorum* Spreng. (0.1% w/v) could cause mortality of citrus red mite (*Panonychus citri* (McGregor)) at 71.10 and 73.53% after 12 and 24 h, respectively. Also, the activities against *P. citri* of the ethanol extracts derived from *Boenninghausenia sessilicarpa* H. Lev., *Laggera pterodonta* (DC.) Benth., *Humulus scandens* (Lour) Merr., and *Rabdosia* were reported with LC_{50} values of 0.9241, 0.9827, 0.9905, and 1.0196 mg/mL, respectively [20]. In addition, applications with aqueous extracts of *Acorus calamus* Linn., *Xanthium strumarium* Linn., *Polygonum hydropiper* Linn., and *Clerodendron infortunatum* (Gaertn.) could lead to more than 50% mortality of *Oligonychus coffeae* (Nietner) [21]. Moreover, 3% methanolic extracts of *Ocimum tenuiflorum* Linn. and *Cassia alata* Linn. exhibited acaricidal activities against *Tetranychus neocaledonicus* Andre. and resulted in the mortality at 93.3 and 97.0%, respectively [22]. On the other hand, 3% aqueous extracts of *C. alata* and *O. tenuiflorum* could lead to mortality of *T. neocaledonicus* at 75% and 82.2%, respectively, after exposure for 3 days. In addition, the volatile oils of *Citrus reticulata* Blanco. and *C. longa* Linn. could cause mortality of *Sitophilus oryzae* Linn. as high as 100 and 90%, respectively [23]. The essential oils of *Ocimum basilicum* Linn., *Coriandrum sativum* Linn., *Eucalyptus globulus* Labill, *Mentha piperita* Linn. and *Satureja hortensis* Linn. were toxic against poultry red mite (*Dermanyssus gallinae* (De Geer)), and, when using the *in vitro* direct contact method, these essential oils at the dose of 0.6 mg/cm could result in mortality rates over 80% after 24 h of contract [24]. Furthermore, *Eucalyptus citriodora* Hook extract was found to be effective against *D. gallinae*, resulting in 85% mortality over a 24 h exposure period in contact toxicity tests [25].

In this study, the rhizome extracts of *C. xanthorrhiza* and *Z. montanum* and the leaf extracts of *O. sanctum* and *M. officinalis* at the dose of 5% (w/v) were found to be highly effective against female adults of *Luciaphorus* sp. The results revealed that the rhizome extracts were likely to have more potent acaricidal activities than those derived from leaves. The acaricidal activities of plant extracts against *Luciaphorus* sp. mites have been previously described. The essential oils derived from lemon grass (*Cymbopogon citratus* Stapf.) and citronella grass (*Cymbopogon nardus* Rendle) were shown to be effective against *Luciaphorus perniciosus* Rack., and the median effective concentration (EC_{50}) was 18.15 and 19.66 ppm, respectively [26]. In addition, the essential oils of *Litsea cubeba* Pers. were effective against *L. perniciosus* by contact and fumigation methods with LD_{50} values equivalent to 0.932 and 0.166 ppm, respectively [27].

In conclusion, the results in this study suggest the possibility of developing plant extracts derived from the rhizomes of *C. xanthorrhiza* and *Z. montanum* and the leaves of *O. sanctum* and *M. officinalis* for controlling *Luciaphorus* mites. The effective concentration and mode of action of these plant extracts against *Luciaphorus* sp. remain to be determined for the future development of highly potent products to be used in the real settings.

Acknowledgments

This work was financially supported by the Thailand Research Fund under Grant number RTA 4880006 and Mahasarakham University. The authors also thank the Department of Biotechnology, Faculty of Technology, Mahasarakham University, Thailand, for providing laboratory equipments and facility.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 309046, 6 pages
doi:10.1155/2012/309046

Research Article

Effect of Crude Leaf Extracts on *Colletotrichum gloeosporioides* (Penz.) Sacc.

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Received 15 April 2012; Accepted 29 May 2012

Academic Editor: Kabkaew Sukontason

Colletotrichum gloeosporioides (Penz.) Sacc. is a fungus that causes anthracnose disease in tropical fruit plants, resulting in damages of the fruit plants and low yield and quality of fruits. The use of chemical fungicides is common for management of this disease, but it also results in the development of fungal resistance to the chemicals. Therefore, this study aims to *in vitro* evaluate the efficacy of 14 crude leaf extracts against *C. gloeosporioides*. The results showed that *Piper sarmentosum* leaf extracts, using 80% of ethanol, methanol, and chloroform as solvents, were found to have very high antifungal activities. Crude methanol extract of *P. sarmentosum* leaves could effectively inhibit the growth of fungal mycelium (100%), followed by crude chloroform extract (81.85%) and 80% ethanol extract (45.50%). Maximum inhibition of *C. gloeosporioides* spore germination could be obtained after application with crude methanol extract of *P. sarmentosum* leaves and crude chloroform extract of *Mentha cordifolia* leaves at 1.25 and 2.5%, respectively. In conclusion, crude extracts of *P. sarmentosum* leaves were found to be highly effective for inhibiting both *C. gloeosporioides* mycelium growth and spore germination, and they have a potential as the new natural fungicides for management of anthracnose disease.

1. Introduction

Colletotrichum gloeosporioides (Penz.) Sacc. is a causative agent for anthracnose disease in many tropical fruit trees such as mango and papaya. This disease is very harmful and can cause spoilage and rotting of fruit plants, resulting in low yield and poor quality of the fruits [1]. The use of chemical fungicides is the most common choice for management of anthracnose disease, but this also causes the development of fungal resistance [2]. In addition, continuous and inappropriate use of chemical fungicides to manage anthracnose disease is not considered to be the long-term solution because this can increase the investment expenses, the risk of having high levels of toxic residues, and also the concerns in human health and environmental settings [3]. Due to these reasons, there are several attempts to search for alternative measures to control the anthracnose disease effectively. Recent efforts have focused on the development of environmentally safe, long-lasting, and effective biocontrol methods for management of anthracnose diseases. The utilization of natural products, especially the plant extracts, has been shown to be effective against many plant pathogens and considered to be safe for consumers and environments [4]. A number of plant species have been reported to possess natural substances that are toxic to a variety of plant pathogenic fungi [5, 6]. The extracts derived from *Curcuma longa* (leaf and rhizome), *Tagetes erecta* (leaf), and *Zingiber officinales* (rhizome) were shown to have antifungal activities against fungal anthracnose by completely inhibiting conidial germination of *C. gloeosporioides* [7]. The aqueous leaf extracts of custard apple (*Annona reticulate* L.) and papaya could inhibit spore formation and germination of *Rhizopus stolonifer* and also conidial formation of *C. gloeosporioides* [8]. In addition, *C. capsici* mycelial growth

and spore germination were found to be suppressed by crude leaf extracts of *Piper betle* L. using methanol, chloroform, and acetone as solvents [9]. Hence, in this study, the *in vitro* antifungal activities of 14 leaf extracts were evaluated against *C. gloeosporioides*, a causative agent of mango anthracnose disease.

2. Materials and Methods

2.1. Fungal Culture. *C. gloeosporioides* was isolated from the upper surface of infected mango and cultured using potato dextrose agar (PDA) medium at 25°C.

2.2. Plant Materials and Extractions. Leaves from 14 different plant species were collected locally or bought at local markets of Maha Sarakham province which is in the northeast region of Thailand (Table 1). Leaf samples were thoroughly washed using tap water, air-dried at room temperature for 3 to 4 h, and finally dried in a hot-air oven at 45–50°C for 1 to 2 days depending on the plant species. Dried leaf samples were ground using small grinder, then placed in polyethylene bags, and stored at 4°C until required. For each sample, 50 g of leaf powder were added to 150 mL of methanol (M), 80% ethanol (E), or chloroform (F) (thus ratio between leaf powder and solvent was 1 : 3). The mixtures were agitated for 72 h on rotary shaker (130 rpm). The obtained extracts were centrifuged at 8,000 rpm for 10 minutes, filtered through Whatman filter paper no. 1, and transferred to 250 mL round-bottom flasks. Finally, these 42 extracts were evaporated using rotary evaporator at 45°C. Concentrated extracts were allowed to dry in hot-air oven, weighed again, and kept at 4°C until required for antifungal assays.

2.3. Screening of Leaf Extracts against C. Gloeosporioides Mycelial Growth. Forty two crude leaf extracts were *in vitro* tested for their efficacy against *C. gloeosporioides* mycelia growth using the poisoned food technique [10]. All crude leaf extracts were reconstituted to have the concentration of 5%. Then 1 mL of each extract was used for mixing with 19 mL of warm PDA and poured into 9 cm sterile Petri dish. After solidification, the plates were inoculated with the 6 mm agar piece containing a week old *C. gloeosporioides* mycelia. For each crude leaf extracts, the experiments were performed in three replicates. PDA plates mixed with carbendazim (commercial fungicide at 0.005%) and sterile distill water were served as positive and negative controls, respectively. The inoculated plates were incubated at 30°C, and the diameters of fungal colonies were measured every day for 5 days.

Inhibition of mycelial growth was calculated using the following formula [11]:

$$\%\ \text{Inhibition} = \frac{X - Y}{X} \times 100, \quad (1)$$

X: diameter of fungal colony grown on negative control plate, Y: diameter of fungal colony grown on plates containing crude leaf extracts.

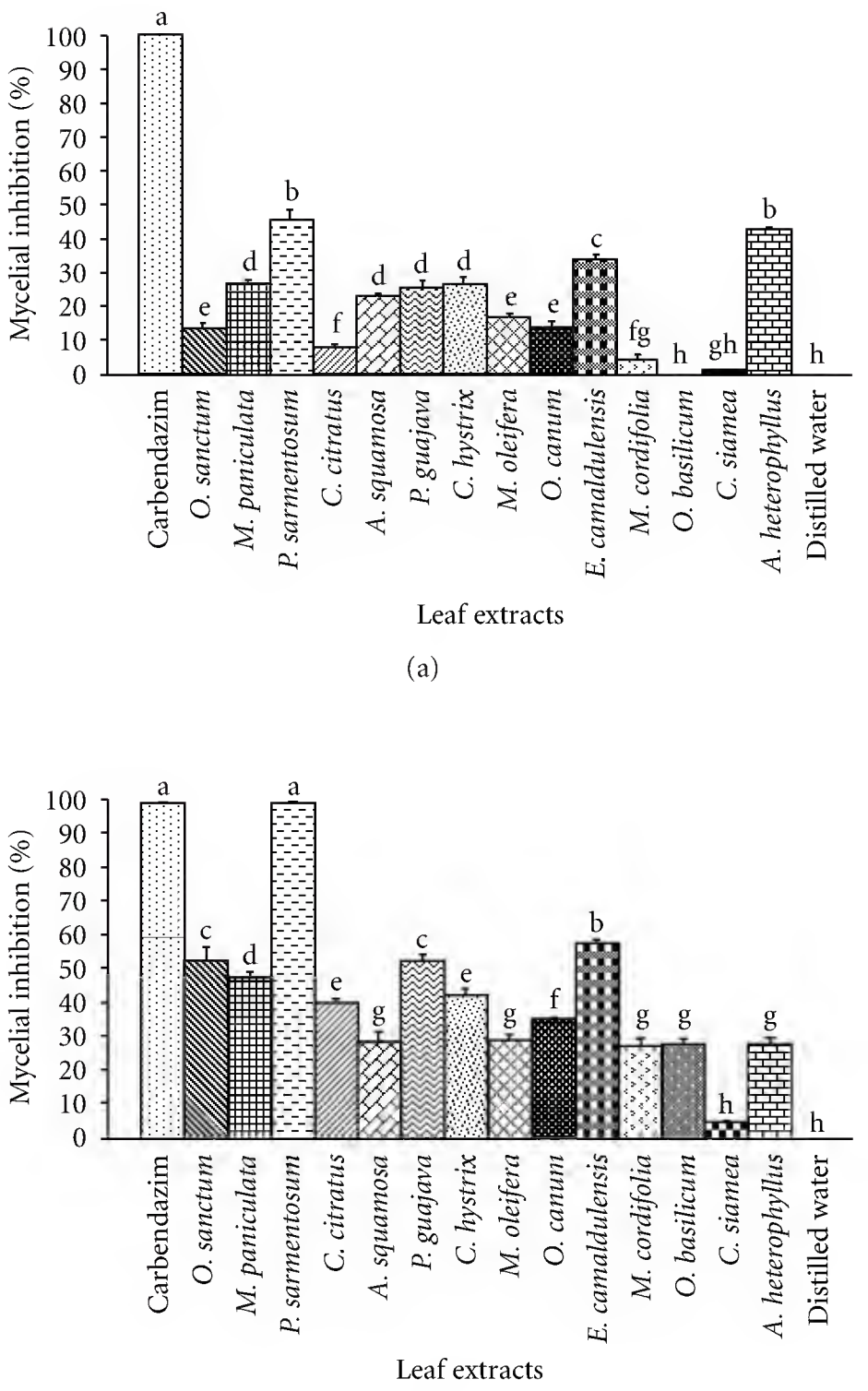

FIGURE 1: Inhibition of *C. gloeosporioides* mycelial growth by crude leaf extracts using (a) 80% ethanol, (b) methanol, and (c) chloroform as solvents. Bars (mean ± SE) with the same letter(s) are not significantly different as determined by LSD test at $P < 0.05$.

Table 1: List of plants.

Scientific name	Family	Common name
Cymbopogon citratus Stapf.	Gramineae	Takhrai, lemongrass
Citrus hystrix DC.	Rutaceae	Leech lime
Murraya paniculata (L.) Jack.	Rutaceae	Orange jessamine, satin-wood
Ocimum basilicum Linn.	Labiatae	Horapa, sweet basil, common basil
Ocimum canum Linn.	Labiatae	Hairy basil
Moringa oleifera Lamk.	Moringaceae	Horse radish tree
Annona squamosa Linn.	Annonaceae	Sugar apple
Ocimum sanctum Linn.	Malvaceae	Holy basil, sacred basil
Psidium guajava Linn.	Myrtaceae	Guava
Eucalyptus camaldulensis Dehnh.	Myrtaceae	Red river gum, red gum
Artocarpus heterophyllus Lam.	Moraceae	Jackfruit tree
Piper sarmentosum Roxb. Ex Hunter.	Piperaceae	Chaplu
Mentha cordifolia Opiz.	Lamiaceae	Kitchen mint, marsh mint
Cassia siamea (Lamk.) Irwin and Barneby	Fabaceae	Cassod tree, siamese senna

2.4. Effect of Leaf Extracts Prepared at Different Concentrations on C. Gloeosporioides Mycelial Growth and Spore Germination. Twelve of the leaf extracts that were found to have high levels of activities against *C. gloeosporioides* mycelial growth were selected for further testing at lower concentrations. Various concentrations of selected crude leaf extracts were prepared (2.5, 1.25, 0.625, 0.3, 0.2, 0.1, and 0.05%) and *in vitro* tested against *C. gloeosporioides* mycelial growth (as described above) and spore germination. Inhibition of spore germination was examined by spreading 100 μL of *C. gloeosporioides* spore suspension (10^5 spores/mL) on PDA plates containing each leaf extracts. Carbendazim and sterile distill water were served as positive and negative controls, respectively. Plates were incubated at 30°C and monitored for 7 days.

2.5. Statistical Analysis. All data were subjected to analysis of variance (ANOVA) using the general linear models procedure (SAS Institute, Cary, NC, USA). The data of the percentages of mycelial inhibition were arcsine transformed before analysis. The means of % mycelial inhibition of all treatments were compared and determined using the LSD test at $P \leq 0.05$.

3. Results

3.1. Screening of 42 Crude Leaf Extracts against C. Gloeosporioides Mycelial Growth. Different solvents used for extraction could result in different levels of *in vitro* antifungal activities of the crude leaf extracts (5%) as measured by poisoned food technique. The antifungal activities of leaf extracts using 80% ethanol, methanol, and chloroform as solvents were found to range between 0.77–45.50%, 4.35–100% and 12.37–100%, respectively (Figure 1). Even though all crude leaf extracts exhibited certain levels of activities against *C. gloeosporioides* mycelia, the 80% ethanol extract of *O. bacilicum* and chloroform extracts of *A. heterophyllus* and *C. siamea* did not effectively prevent mycelial growth. *C. siamea* leaves that were extracted using 80% ethanol and methanol were found to have very low antifungal activities at 0.77 and 4.35%, respectively (Figure 1).

Crude leaf extracts using 80% ethanol as solvent were shown to have rather low antifungal activities (less than 50%), as shown by that *P. sarmentosum, A. heterophyllus,* and *E. camaldulensis* could prevent the growth of *C. gloeosporiodes* mycelia at 45.50, 42.75, and 33.85%, respectively (Figure 1(a)). Interestingly, crude methanol extracts of *P. sarmentosum* leaves exhibited the highest inhibition activities against *C. gloeosporioides* mycelialgrowth (100%), followed by *E. camaldulensis* (57.75%), *O. sanctum* (52.75%), and *P. guajava* (52.75%). However, the other methanol leaf extracts were found to have levels of antifungal activities less than 50% (Figure 1(b)). There were 5 chloroform extracts that were found to have more than 50% inhibition activities against *C. gloeosporioides* mycelial growth, including *M. cordifolia* (100%), *P. sarmentosum* (81.75%), *E. camaldulensis* (60.25%), *M. paniculata* (55.50%), and *O. bacilicum* (91.10%) (Figure 1(c)).

3.2. Effect of Leaf Extracts Prepared at Different Concentrations on C. Gloeosporioides Mycelial Growth and Spore Germination. Twelve crude leaf extracts (derived from 7 plant species), including the extracts of *P. sarmentosum* and *E. camaldulensis* in all solvents, the extract of *A. heterophyllus* in 80% ethanol, the extracts of *O. sanctum* and *P. guajava* in methanol, and the extracts of *O. bacillicum* and *M. paniculata* in chloroform were prepared at various concentrations (2.5, 1.25, 0.625, 0.3, 0.2, 0.1, and 0.05%) and determined for their efficacy against *C. gloeosporioides* mycelia growth and spore germination.

Although at lower concentrations these plant extracts exhibited lower antifungal activities, some plant extracts remained effective (Tables 2 and 3). In particular, when compared to carbendazim (commercial fungicide), the crude methanol extract of *P. sarmentosum* and chloroform extract of *M. cordifolia* at 2.5% could significantly inhibit the

Table 2: Effect of 12 selected crude leaf extracts prepared at different concentrations on *Colletotrichum gloeosporioides* mycelial growth.

Leaf extracts	% inhibition of *Colletotrichum gloeosporioides* mycelial growth* Concentration (%)						
	2.5	1.25	0.625	0.3	0.2	0.1	0.05
M. paniculata/C	54.45 ± 0.71^{cA}	52.75 ± 0.35^{efB}	33.40 ± 0.85^{fC}	17.25 ± 1.06^{fD}	16.50 ± 2.12^{cD}	0.57 ± 0.18^{cdE}	0.00 ± 0.00^{dE}
A. heterophyllus/E	7.45 ± 1.10^{hA}	3.85 ± 0.50^{jkB}	1.95 ± 0.78^{jkC}	0.27 ± 0.03^{hD}	0.00 ± 0.00^{fD}	0.00 ± 0.00^{eD}	0.00 ± 0.00^{dD}
O. sanctum/M	3.85 ± 0.21^{iA}	1.12 ± 0.53^{klB}	0.00 ± 0.00^{kC}	0.00 ± 0.00^{hC}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{eC}	0.00 ± 0.00^{dC}
P. guajava/M	35.25 ± 1.06^{eA}	20.50 ± 0.70^{iB}	0.00 ± 0.00^{kC}	0.00 ± 0.00^{hC}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{eC}	0.00 ± 0.00^{dC}
O. basilicum/C	57.25 ± 1.06^{cA}	56.50 ± 0.70^{eA}	36.35 ± 2.33^{eB}	21.50 ± 0.7^{eC}	9.87 ± 0.18^{dD}	0.37 ± 0.17^{deE}	0.00 ± 0.00^{dE}
M. cordifolia/C	97.60 ± 0.84^{aA}	75.75 ± 1.76^{cB}	56.50 ± 2.12^{cC}	37.50 ± 0.7^{cD}	19.12 ± 1.24^{bE}	3.85 ± 0.50^{bF}	37.50 ± 0.71^{cG}
P. sarmentosum/E	16.50 ± 2.12^{gA}	5.30 ± 0.98^{jB}	3.35 ± 0.92^{jB}	0.27 ± 0.03^{C}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{eC}	0.00 ± 0.00^{dC}
P. sarmentosum/M	100.00 ± 0.00^{aA}	88.25 ± 1.76^{bB}	80.00 ± 1.41^{bC}	56.50 ± 0.71^{bD}	19.10 ± 2.70^{bE}	3.85 ± 0.51^{bF}	1.37 ± 0.10^{bF}
P. sarmentosum/C	77.75 ± 1.80^{bA}	68.00 ± 2.83^{dB}	42.25 ± 1.06^{dC}	30.85 ± 1.63^{dD}	9.50 ± 2.12^{dE}	0.00 ± 0.00^{eF}	0.00 ± 0.00^{dF}
E. camaldulensis/E	29.75 ± 2.48^{fA}	26.50 ± 4.95^{hB}	23.85 ± 0.21^{gC}	16.75 ± 0.35^{fD}	5.85 ± 0.51^{eE}	0.82 ± 0.71^{cF}	0.00 ± 0.00^{dF}
E. camaldulensis/M	49.50 ± 2.12^{dA}	37.25 ± 1.06^{gB}	11.50 ± 2.12^{iC}	8.85 ± 0.50^{gCD}	7.75 ± 0.35^{deD}	0.00 ± 0.00^{eE}	0.00 ± 0.00^{dE}
E. camaldulensis/C	55.25 ± 2.50^{cA}	50.75 ± 1.06^{fB}	15.25 ± 2.47^{hC}	8.35 ± 0.50^{gD}	1.37 ± 0.11^{fE}	0.00 ± 0.00^{eE}	0.00 ± 0.00^{dE}
Carbendazim (0.005%)	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}
Distilled water	0.00 ± 0.00^{jA}	0.00 ± 0.00^{lA}	0.00 ± 0.00^{kA}	0.00 ± 0.00^{hA}	0.00 ± 0.00^{fA}	0.00 ± 0.00^{eA}	0.00 ± 0.00^{dA}

C: chloroform, M: methanol, E: 80% ethanol.
*Percentages of inhibition within the row followed by the same uppercase letter are not significantly different at $P < 0.05$ as determined by LSD test. Percentages of inhibition within the column followed by the same lowercase letter are not significantly different at $P < 0.05$ as determined by LSD test.

Table 3: Effect of 12 selected crude leaf extracts prepared at different concentrations on *Colletotrichum gloeosporioides* spore germination.

Leaf extracts	% inhibition of *Colletotrichum gloeosporioides* spore germination* Concentration (%)						
	2.5	1.25	0.625	0.3	0.2	0.1	0.05
M. paniculata/C	51.10 ± 2.68^{cdA}	42.00 ± 4.24^{deB}	32.35 ± 5.16^{efC}	16.25 ± 3.88^{eD}	13.00 ± 2.82^{eD}	5.00 ± 1.41^{efE}	0.00 ± 0.00^{eE}
A. heterophyllus/E	42.50 ± 2.12^{eA}	38.50 ± 3.50^{efA}	21.50 ± 0.71^{gB}	13.20 ± 2.54^{eC}	4.50 ± 0.71^{fgD}	0.00 ± 0.00^{fE}	0.00 ± 0.00^{eE}
O. sanctum/M	33.35 ± 1.06^{fA}	24.00 ± 1.41^{gB}	0.00 ± 0.00^{hC}	0.00 ± 0.00^{eC}	10.00 ± 0.00^{gC}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{eC}
P. guajava/M	50.66 ± 1.83^{cdA}	35.25 ± 5.39^{fB}	0.00 ± 0.00^{hC}	0.00 ± 0.00^{eC}	0.00 ± 0.00^{gC}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{eC}
O. basilicum/C	65.00 ± 7.07^{bA}	58.60 ± 1.97^{cAB}	47.20 ± 5.37^{dBC}	36.33 ± 0.95^{dC}	22.00 ± 1.41^{dC}	9.00 ± 0.00^{eD}	0.00 ± 0.00^{eD}
M. cordifolia/C	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	85.30 ± 6.64^{bB}	67.00 ± 8.48^{bC}	59.33 ± 3.74^{bC}	42.50 ± 0.71^{cD}	31.12 ± 2.65^{cE}
P. sarmentosum/E	56.75 ± 3.88^{cA}	48.00 ± 2.83^{dB}	37.30 ± 1.83^{eC}	20.00 ± 2.83^{eD}	12.20 ± 1.13^{eE}	8.00 ± 1.42^{eE}	0.00 ± 0.00^{eF}
P. sarmentosum/M	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	90.50 ± 6.36^{bB}	72.20 ± 5.94^{bC}	65.15 ± 6.85^{bD}	57.00 ± 8.48^{bD}	36.10 ± 5.51^{bE}
P. sarmentosum/C	100.00 ± 0.00^{aA}	87.50 ± 2.12^{bB}	66.70 ± 3.25^{cC}	55.60 ± 1.97^{cD}	28.00 ± 5.65^{cE}	19.12 ± 1.24^{dF}	14.00 ± 5.66^{dF}
E. camaldulensis/E	48.10 ± 5.15^{deA}	41.60 ± 4.80^{efA}	31.20 ± 1.13^{efB}	18.00 ± 1.41^{eD}	10.50 ± 0.71^{eD}	4.00 ± 1.42^{efDE}	0.00 ± 0.00^{eE}
E. camaldulensis/M	54.35 ± 5.16^{cdA}	43.33 ± 2.36^{deB}	29.50 ± 0.71^{fC}	18.20 ± 1.13^{eD}	10.00 ± 1.41^{efE}	0.00 ± 0.00^{fF}	0.00 ± 0.00^{eF}
E. camaldulensis/C	47.30 ± 3.81^{deA}	40.00 ± 4.24^{efA}	28.16 ± 2.60^{fgB}	17.00 ± 1.41^{eBC}	8.00 ± 0.00^{efCD}	0.00 ± 0.00^{fD}	0.00 ± 0.00^{eD}
Carbendazim (0.005%)	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}
Distilled water	0.00 ± 0.00^{gA}	0.00 ± 0.00^{hA}	0.00 ± 0.00^{hA}	0.00 ± 0.00^{fA}	0.00 ± 0.00^{gA}	0.00 ± 0.00^{fA}	0.00 ± 0.00^{eA}

C: chloroform, M: methanol, E: 80% ethanol.
*Percentages of inhibition within the row followed by the same uppercase letter are not significantly different at $P < 0.05$ as determined by LSD test. Percentages of inhibition within the column followed by the same lowercase letter are not significantly different at $P < 0.05$ as determined by LSD test.

growth of *C. gloeosporioides* mycelium at 100 and 97.60%, respectively (Table 2); moreover, both of these plant extracts at 1.25% also completely prevented *C. gloeosporioides* spore germination (100%) (Table 3). However, the other crude leaf extracts at lower concentrations did not exhibit significant antifungal activity against *C. gloeosporioides* (Tables 2 and 3).

4. Discussion

In this study, 12 leaf extracts (obtained from 7 plant species) that were found to inhibit *C. gloeosporioides* mycelial growth very strongly at 5% were selected, then prepared at lower concentrations, and used for further evaluation against

C. gloeosporioides mycelial growth and spore germination (Tables 2 and 3). At 2.5%, the crude methanol extract of *P. sarmentosum* and chloroform extract of *M. cordifolia* were shown to inhibit *C. gloeosporioides* mycelial growth, and at 1.25%, both of these could also completely prevent *C. gloeosporioides* spore germination.

From previous reports, there are a variety of plant extracts that were used to control fungal anthracnose. For instance, crude methanol, chloroform, and acetone extracts of *Piper betle* leaves at the concentration of 10 μg/mL could inhibit the growth of *Colletotrichum capsici* (responsible for anthracnose disease in pepper) mycelium at 85.25, 78.53, and 73.58%, respectively [9]. Also, at the same concentration, crude methanol, chloroform, and acetone extracts of these *P. betle* leaves were found to prevent *C. capsici* spore germination at 80.93, 74.09, and 72.91%, respectively [9]. Moreover, the leaf extracts of *O. bacillicum* and *Allium sativum* exhibited 100% inhibition of *C. gloeosporioides* (responsible for anthracnose in para rubber) mycelial growth when applying at 50 and 100% w/v, respectively, and both of these extracts could completely suppress spore germination when applying as minimal as 10% w/v [12]. Furthermore, the ethanol extracts of *Ocimum gratissimum* and *Aframomum melegueta* leaves were shown to inhibit the growth of *Botryodiplodia theobromae* mycelium (causative agent of banana anthracnose) at 72.1 and 68.2%, respectively [13].

Other plant pathogenic fungi could also be inhibited by plant extracts. For example, the ethanol extracts of *O. gratissimum* and *A. melegueta* leaves were also reported to prevent *Fusarium oxysporum* and *Aspergillus niger* spore germination at over 65% [13]. In addition, *Rhizopus oryzae* spore germination and mycelia growth were found to be suppressed by the leaf extracts of *O. gratissimum* [14].

This study showed that *P. sarmentosum* and *M. cordifolia* leaves had significant antifungal activity. The studies of phytochemical characteristics showed that bioactive compounds in *Mentha* sp. are sitosterol and β-sitosteryl-β-D-glucoside, and in *Piper* sp. are lignans, steroids, neolignans, alkaloids, propenylphenols, terpenes, piperolides, chalcones, flavanones, flavones, and amides bearing isobutyl, pyrrolidine, dihydropyridine, and piperidine moieties, all of which could exhibit high antimicrobial and antifungal properties [15–17]. The levels of plant bioactive compounds with antifungal activity could be influenced by many factors which include the age of plant, harvesting time point, extraction solvent, and method of extraction [18].

In conclusion, this study shows that crude leaf extracts of *P. sarmentosum* have strong antifungal activities against *C. gloeosporioides*. This may suggest their potential for future formulation into products for controlling anthracnose diseases of mango and other fruits. More extensive study of their phytochemical characteristics and *in vivo* efficacy remains to be determined.

Acknowledgments

This work was financially supported by the NRCT, Thailand. The authors also thank the Department of Biotechnology, Faculty of Technology, Mahasarakham University, Thailand, for laboratory equipments and facility.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 535062, 14 pages
doi:10.1155/2012/535062

Research Article

Impact of the Invasive Brown Marmorated Stink Bug, *Halyomorpha halys* (Stål), in Mid-Atlantic Tree Fruit Orchards in the United States: Case Studies of Commercial Management

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Received 11 January 2012; Accepted 2 May 2012

Academic Editor: Jeffrey R. Aldrich

Four commercial orchards in the mid-Atlantic region of the United States were surveyed weekly in 2010 and 2011 for the presence of brown marmorated stink bug and the injury caused to both apple and peaches. Among tested sampling techniques, pyramid traps baited with the aggregation pheromone of *Plautia stali* Scott, methyl-(2*E*,4*E*,6*Z*)-decatrienoate, yielded the most brown marmorated stink bug adults and nymphs, followed by visual observations. Brown marmorated stink bugs began to feed on apples and peaches soon after fruit set and continued to feed on fruit throughout the growing season. Injury to apple was relatively inconsequential until after mid-June, whereas feeding on peaches resulted in immediate economic injury as the surface became distorted, dented, discolored, and the flesh beneath turned brown. Significantly more apples were injured and with greater severity in 2010 than in 2011. Likewise, percent injury on the exterior portion of each apple plot was significantly greater than injury reported from the interior in both years. Growers increased the number of insecticide applications nearly 4-fold from 2010 to 2011. In addition to the increased number of targeted insecticide applications, growers also reduced the interval between treatments in 2011. A metric was created to compare the relative intensity of each grower's commercial management program between seasons and amongst each other.

1. Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Stål), is an invasive stink bug native to Japan, Korea, China, and Taiwan [1], now well established throughout the mid-Atlantic region of the United States. Evidence of established populations in Switzerland [2] and Canada [3] has also been reported. Brown marmorated stink bug is an extremely polyphagous species, and a pest of many crops in Asia [4] including tree fruit, vegetables, shade trees, and leguminous crops with specific mention of apple, cherry, peach, and pear [4, 5]. Surveys conducted in the United States identified a number of tree fruit hosts for brown marmorated stink bug including apple, plum, peach, pear, and cherry [5–7]. In 2010, populations of this invasive species increased dramatically, causing widespread injury to many crops throughout the mid-Atlantic region [8]. Tree fruit, in particular, was hit hard with some growers losing entire crops of stone fruit. Among apple growers, losses were totaled in excess of 37 million dollars in the region [9].

Within the United States, native stink bugs generally have been classified as secondary pests of tree fruit orchards and have been successfully managed with broad-spectrum insecticide applications typically directed at other key pests. However, with the passage of the Food Quality Protection Act in 1996, many broad-spectrum materials have been eliminated or severely curtailed for use through regulatory measures. Subsequently, populations of native stink bugs, long considered to be secondary pests, became more prevalent in orchard agroecosystems [10, 11]. Furthermore, when brown marmorated stink bug populations increased dramatically, this led to devastating levels of fruit injury as

this invasive species quickly replaced lepidopteran pests such as codling moth, *Cydia pomonella* L., and oriental fruit moth, *Grapholita molesta* (Busck), as the key pest driving management decisions in the mid-Atlantic region of the United States.

Because brown marmorated stink bug is a newly established invasive species, management programs for this pest are still being developed. In 2010, no specific management recommendations were in place and only a single laboratory study evaluating a select number of compounds against brown marmorated stink bug had been conducted [12]. Growers were forced to rely on recommendations made for native stink bugs, which did not result in satisfactory control [8]. In general, pyrethroid insecticides, considered to be effective against native stink bugs, but are a poor fit in IPM programs because of their negative impact on beneficial arthropods [13, 14] were applied.

Recent insecticide trials against brown marmorated stink bug have revealed that numerous pyrethroid and neonicotinoid compounds at field-labeled rates are not particularly effective, with many compounds resulting in greater than 33% of the individuals recovering from a moribund or "knockdown" state [15]. This conforms to earlier laboratory [12] and field studies [16] that documented knockdown and recovery from pyrethroids specifically. On the other hand, there are a number of materials labeled for either stone or pome fruit that resulted in substantial mortality of tested individuals. Some effective materials reported in the previous study were endosulfan, a chlorinated hydrocarbon; malathion, an organophosphate; permethrin and fenpropathrin, pyrethroids; dinotefuran, a neonicotinoid; methomyl, a carbamate [15]. Prior to the establishment of brown marmorated stink bug, growers likely would not have applied many of these materials in their management programs as they were not needed to achieve acceptable levels of control of other key pests. However, management programs have rapidly evolved to meet the challenge posed by brown marmorated stink bug.

Simultaneously, development of monitoring tools that can be used to assess the presence, abundance, and seasonal activity of this invasive species is considered paramount [8]. Stink bug species are typically monitored in cropping systems using sweep nets, beating samples, pheromone-baited traps, and/or black light traps. Among native stink bugs in tree fruit, baited yellow pyramid traps [10, 11] and baited mullein plants [17] were effective at monitoring native *Euschistus* spp. while *Chinavia hilaris* (Say) was monitored in vegetable and row crops using black light traps [18]. Black light traps have been evaluated for brown marmorated stink bug in Japan [19] and in New Jersey [6]. Most recently, black pyramid traps baited with the aggregation pheromone of *Plautia stali* Scott, methyl-(2*E*,4*E*,6*Z*)-decatrienoate [20], were found to be an effective means to trap brown marmorated stink bug adults and nymphs [21, 22]. However, none of these tools have been evaluated extensively against brown marmorated stink bug in commercial tree fruit orchards.

In 2010 and 2011, we surveyed commercial fruit orchards in the mid-Atlantic to quantify the amount and severity of injury to stone and pome fruit crops. We also evaluated the efficacy of established monitoring techniques for other stink bug species to measure presence, abundance, and seasonal activity of populations of brown marmorated stink bug. Finally, we quantified the changes in management programs from 2010 to 2011 in terms of material selection, interval, and application method.

2. Materials and Methods

2.1. Commercial Site Selection. We attempted to evaluate the population density of brown marmorated stink bug and severity of injury to apple and peach fruit in two West Virginia and two Maryland orchards. In 2010, the project began during mid-season (July) and continued through November. Grower orchards were selected based on (1) the presence of brown marmorated stink bug infestations and (2) the availability of both apples and peaches as hosts. Specific apple and peach plots within orchards were chosen based on grower reports of stink bug injury and close proximity (<20 m) to wooded/wild habitats. Orchard A consisted of a 2.9 ha apple orchard ("Fuji" on M7A; Spacing: 4.9 m × 7.3 m) planted in 1995 and a 1.3 ha peach orchard ("Redhaven" on Tenn. Nat. and "Sentry" and "Bounty" on Lovell; Spacing: 4.3 m × 7.3 m) planted in 2001. Orchard B consisted of a 5.7 ha apple orchard ("Delicious" and "Golden Delicious" on M111; Spacing: 6.2 m × 8.6 m) planted in 1996 and a 5.3 ha peach orchard ("Sunbright" on Lovell; Spacing: 6.2 m × 7.4 m) planted in 1996. Orchard C consisted of 1.9 ha apple orchard ("Golden Delicious" on M111; Spacing: 4.2 m × 7.6 m) planted in 1975 and a 0.7 ha peach orchard ("Red Haven" and "Blake" on Lovell; Spacing: 4.2 m × 7.6 m) planted in 1997. Orchard D consisted of a 1.8 ha apple orchard ("Delicious" on M26, "Fuji", "Braeburn", "Mutsu", "Empire", "Jonagold" and "Magnolia Gold" on M9; Spacing: 2.7 m × 4.0 m) planted in 1992 and a 2.4 ha peach orchard (mostly "Loring", "Cresthaven", "Encore", "White Lady" and "Redhaven" on Lovell, "Beekman" on Tenn. Nat. and "John Boy" on Guardian; Spacing: 5.2 m × 6.1 m) planted in 1998. In 2011, the same four growers' orchards were monitored and evaluated for brown marmorated stink bug presence and injury to fruit throughout the entire growing season (April–November). The only exception was at orchard C where the apple plot was 1.8 ha ("Fuji" and "York" on M111/M9 interstem; Spacing: 3.0 m × 6.1 m) planted in 2001 and the peach plot was 1.9 ha (mostly "Canadian Harmony", "Bounty", and "Blake" on Lovell; Spacing: 3.7 m × 6.1 m) planted in 2003.

2.2. Aggregate Insecticide Metric (AIM). Growers selected their own spray programs for both seasons; however, growers used more targeted treatments against brown marmorated stink bug in 2011. In order to assess the insecticide treatment programs used in 2010 and 2011, a metric (AIM) was created that would compare quantitatively the differences in material and application method for each chemical used. The AIM takes into account the lethality of the active ingredient against brown marmorated stink bug [15], the number of insect Orders listed as controlled on each product label, the proportion of chemical used by the grower versus the

maximum allowed according to approved label directions, and the method of application (complete block or alternate row middle).

2.2.1. Material. Each material/active ingredient was compared by a series of three steps: (1) general insect toxicity, (2) specific brown marmorated stink bug toxicity, and (3) amount of active ingredient used. First, general insect toxicity (G_i) was assessed by counting the number of insect Orders presumed (according to the specific product label) to be controlled upon use of the chemical, then dividing that number by the number of insect Orders available for control and presented as a proportion (0 to 1). The identified Orders of insects available for control were Coleoptera, Diptera, Hemiptera, Homoptera, Hymenoptera, Lepidoptera, Orthoptera, and Thysanoptera [23]. Specific brown marmorated stink bug toxicity (S_i) was evaluated among chemicals by use of the lethality index reported in Leskey et al. [15]. This index was based on the results from laboratory tests on adult brown marmorated stink bugs exposed to high field-rate doses of various active ingredients, presented as dislodgeable, dry residues for a period of 4.5 h. Subsequently, all test subjects were evaluated daily over a 7-d period for their condition (alive, moribund, or dead). These data comprise the lethality index, which assigned a value 0 to 100 based on the speed and efficacy at which a chemical acted against the brown marmorated stink bug; however, in this publication the lethality index was assessed from 0 to 1 to standardize with other factors in the model. Increased efficacy yielded a higher number and vice versa. In the third examination of each material, the amount of active ingredient (A_i) used in each application was calculated as a proportion (0 to 1) of the amount of material the label permits per application. Thus the amount of material used was divided by the maximum amount that could have been used.

2.2.2. Application Method (M_i). All growers used one of two methods for applying pesticides to their fruit trees: complete block or alternate row middle sprays [24]. In the complete block spray, chemicals were applied to every tree from every drive row within a plot. In the alternate row middle spray, chemicals were applied to one half of every tree via the use of every other drive row within a plot. A complete block spray was assigned a value of 1 whereas an alternate row middle spray was assigned a value of 0.5.

2.2.3. AIM Formula. The AIM value for each insecticide application was calculated by multiplying each factor:

$$G_i \times S_i \times A_i \times M_i. \quad (1)$$

For each grower by year and fruit species, we calculated the mean AIM and total AIM. Given that grower management programs were not limited to control of brown marmorated stink bug, statistical comparisons of the mean interval between applications and the mean AIM score was computed for all growers combined. These means were compared using Student's t-test ($P < 0.05$).

2.3. Sampling/Monitoring. From 12 May to 20 October 2011, two commercial orchards (A & C) were monitored weekly for brown marmorated stink bug presence by the use of three sampling techniques: sweep net, limb jarring, and visual surveying. Each sampling technique was performed in both apple [12 May to 6 October (orchard A) and from 12 May to 20 October(orchard C)] and peach (12 May to 7 July) plots at each orchard.

2.3.1. Sweep Net Samples. Three areas were sampled at the border of the wooded/wild habitat proximal to each orchard plot and spaced equidistant to span the length of the plot. Fifty sweeps of the ground flora, consisting of a back-and-forth motion, were performed weekly in each area covering approximately 5 m^2. The number of nymphs and adults collected were taken to the laboratory and counted.

2.3.2. Limb Jarring Samples. Eight apple and peach trees were selected from the perimeter row of each plot that bordered a wooded/wild habitat and were then revisited each week for subsequent samples. Two limbs on opposing sides were sampled by striking each limb three times onto a 1 m^2 canvas beating sheet (BioQuip, Rancho Dominguez, CA) to collect dislodged insects. All nymphs and adults on the sheet were counted and totaled for each tree.

2.3.3. Visual Surveys. Eight additional apple and peach trees were selected from the perimeter row of each plot that bordered a wooded/wild habitat and were then revisited each week for subsequent samples. Each sample consisted of a 3-min visual inspection of all parts of the tree. All brown marmorated stink bug eggs (hatched and unhatched), nymphs, and adults were counted and any hatched eggs discovered were removed from the tree.

2.3.4. Trapping. On 4 August 2010, three black pyramid traps [21] were deployed in the perimeter tree row of apple plots at each commercial orchard. All traps were placed along the perimeter that bordered a wooded/wild habitat. In 2010, traps were baited with 50 mg of methyl-(2*E*,4*E*,6*Z*)-decatrienoate (ChemTica Intl., Atlanta, GA), an aggregation pheromone of *Plautia stali* Scott [20] and a known cross-attractant to the brown marmorated stink bug [22]. Traps were also provisioned with an insecticidal strip containing 10% 2,2-dichlorovinyl dimethyl phosphate (Vaportape II, Hercon, Emigsville, PA) to inhibit stink bug escape from the trap. The chemical attractant and insecticidal strip were replaced at 4-wk intervals. Brown marmorated stink bug adults captured in traps were sexed, and nymphs were separated by instar and then removed from the trap weekly until 10 November. In 2011, a prototype trap developed by AgBio (Westminster, CO), patterned after the 2010 trap, was used in both apple and peach blocks at the four commercial orchards described previously. The pyramid base was constructed from 2 sheets of laminated plywood joined together with glue and staples. The trap was painted with flat black exterior latex paint and was 1.1 m tall × 0.5 m wide at base × 0.64 cm thick (Figure 1(a)). A 1.9 L plastic jar

(a) (b)

Figure 1: (a) Photo of pyramid trap and (b) jar top used in commercial orchards in 2011.

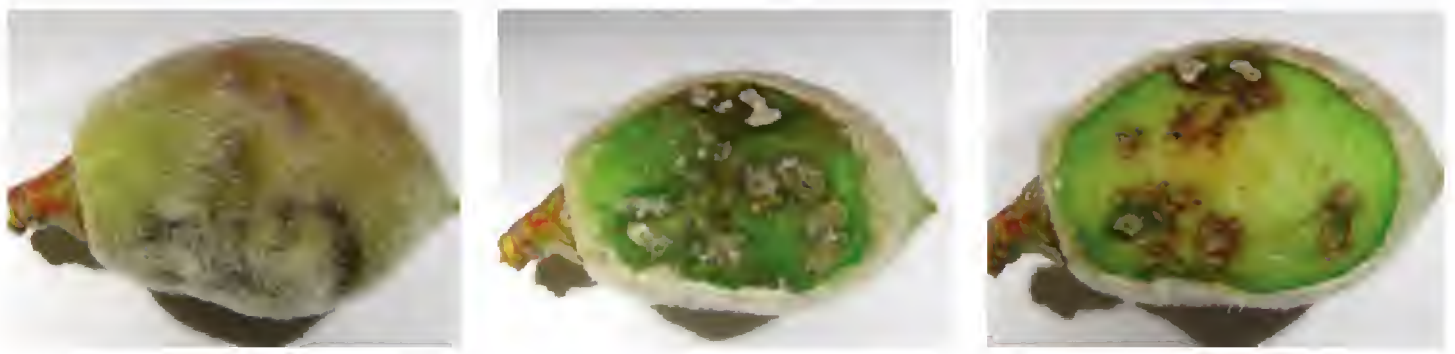
top was fixed atop a yellow plastic funnel with an opening of approximately 2.5 cm, which served as the entry point at the base of the jar. The funnel was not permanently attached to the jar top because its contents were accessed by separation of the jar top from the funnel. A hole was drilled into each edge of the pyramid trap so that the funnel and jar could be held in place at the top of the pyramid by four 5.1 cm, 6-penny nails pushed into the sides of the trap. The four sides of the jar top contained 23 holes, 1 mm in diameter, spaced 1 cm apart over 14 sq·cm (Figure 1(b)). The same lure and insecticidal strips were used as in 2010, except the load was increased to 66 mg. Placement of traps and sampling protocols were similar to those used in 2010, with only sampling period duration differing. Traps were sampled weekly from 8 April (Orchards A–C) and 15 April (Orchard D) through 18 November. The number of brown marmorated stink bugs captured per week from 4 August to 12 November was compared between years with Student's *t*-test. The change in the ratio of adults: nymphs captured in apples in 2010 at each orchard was compared with a chi-square test. The same test was not performed in 2011 due to limited captures in all orchards.

2.4. Injury Assessments. In 2010, fruit were evaluated weekly from 30 July to harvest, relative to each cultivar, for the presence of stink bug damage in apples and peaches. One hundred apples and one hundred peaches were picked from both the exterior and interior at each commercial orchard. The exterior was limited to the three outermost rows of each plot and was bordered by a wooded/wild habitat; while interior fruit were selected from the middle third of each plot. The surface of each fruit was visually examined and the side of the fruit appearing to have the greatest number of injury sites was sectioned to the core. The total number of injured fruit and independent injury sites on one side of the fruit, indicated by the presence of subsurface corking (Figures 2 and 3(b)), was recorded. In 2011, fruit evaluations were conducted weekly from 18 May to harvest of each cultivar using a similar protocol to that established in 2010. Evaluations were conducted prior to 18 May as on-tree visual samples of the surface of both 100 apples and peaches, but proved too unreliable to accurately assess the level of injury and so all subsequent evaluations involved removal of fruit from the tree. Samples prior to 18 May will not be reported in this paper. Thereafter, 200 peaches and 100 apples were destructively sampled weekly from the exterior of each plot. The peach evaluation was the same as that in 2010. This level of recording persisted until 13 July, where the protocol returned to that of 2010. Due to variation in expression of injury in apple relative to fruit maturity, the entire surface of each apple was evaluated for the presence of a feeding hole or dimple until apple injury was expressed as a depression or discolored depression [25]. At this time, fruit were sectioned to the core, and the total number of injured fruit, based on the presence of corking in the flesh (Figure 3), was recorded. In 2011, only the exterior of each apple and peach plot was sampled until 5% of fruit contained at least one subsurface corky spot. Once an interior sample was triggered, only 100 fruit of each species were sampled. Thus, from August on, fruit were evaluated for the presence of corking in the flesh and the number of individual corking spots. Percent corking injury and number of injury sites per injured fruit on the exterior and interior of plots were compared using a Student's *t*-test and percentages were arcsin-square root transformed as needed.

3. Results

3.1. Aggregate Insecticide Metric

3.1.1. Apple. At all four commercial orchards, growers increased the number of brown marmorated stink bug-targeted insecticide applications and decreased the time between



(a)

(b)

FIGURE 2: (a) Photos of subsurface corking injury to a 15 mm "Loring" peach in the early season and to (b) a 40 mm "Red Haven" peach ~3 weeks prior to harvest.

(a) (b)

FIGURE 3: (a) Photo of subsurface feeding sheath that is the result of feeding in the early season on "Golden Delicious" apple and of (b) subsurface corking injury on "Turley Winesap" which is the result of feeding taking place later in the season (from ~6–8 weeks after petal fall until harvest).

consecutive applications from 2010 to 2011 ($t = 5.67$; df = 118; $P < 0.0001$). The total AIM score increased numerically from 2010 to 2011, but there was no statistical difference in the mean AIM score ($t = 1.078$; df = 150; $P = 0.2827$) (Table 1).

3.1.2. Peach. At all four commercial orchards, growers increased the number of brown marmorated stink bug-targeted insecticide applications and decreased the time between consecutive applications ($t = 3.45$; df = 86; $P = 0.0009$). The mean AIM score increased significantly ($t = 2.486$; df = 109; $P = 0.0144$) and total AIM score also increased from 2010 to 2011 (Table 2).

3.2. Sampling/Monitoring

3.2.1. Apple. Orchard A yielded a total of 12 brown marmorated stink bugs in limb jarring, 21 in sweep net, and 77 in visual samples season-long. At orchard C, 9 brown marmorated stink bugs were collected in limb jarring samples, 14 from sweep nets, and 49 in visual observations season long (Figure 4(a)).

3.2.2. Peach. At orchard A, a total of 3 brown marmorated stink bugs were recovered from limb jarring samples, 4 from visual observations and 0 from sweep net ground samples season-long. No brown marmorated stink bugs were

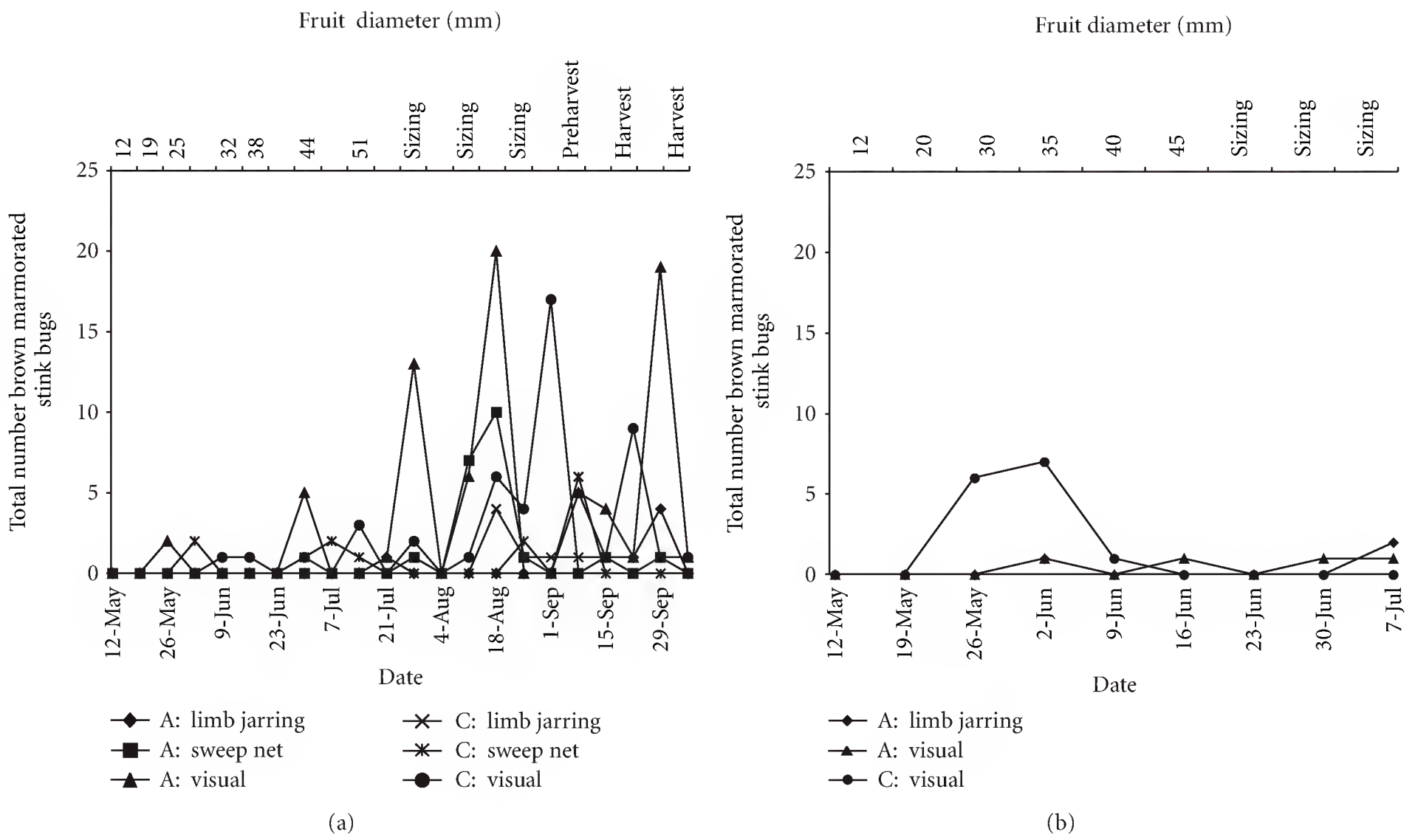


FIGURE 4: (a) Total number of brown marmorated stink bug eggs, nymphs, and adults recovered from limb jarring, sweep net, and visual samples in apple trees and (b) the total number recovered from limb jarring and visual samples in peach trees at orchard A and C in 2011.

TABLE 1: Total number of targeted brown marmorated stink bug insecticide applications, mean interval (d) ± SEM between insecticide applications, and mean A.I.M. score ± SEM and total A.I.M. score in apple plots from 2010 to 2011 at four commercial orchards.

Orchard	Number of targeted insecticide applications		Mean insecticide interval ± SEM*		A.I.M. score Mean ± SEM*		A.I.M. score Total	
	2010	2011	2010	2011	2010	2011	2010	2011
A	3	20	10.6 ± 1.9	7.2 ± 0.4	0.06 ± 0.02	0.19 ± 0.02	0.89	5.26
B	5	7	22.2 ± 5.7	18.8 ± 2.5	0.40 ± 0.10	0.46 ± 0.10	3.63	5.47
C	4	12	18.5 ± 1.3	11.4 ± 1.4	0.18 ± 0.05	0.29 ± 0.06	1.78	5.31
D	7	42	10.6 ± 1.0	4.1 ± 0.3	0.21 ± 0.05	0.18 ± 0.02	3.28	8.16
All Orchards	19	81	14.0 ± 1.3 a	7.1 ± 0.6 b	0.20 ± 0.03 a	0.24 ± 0.02 a	9.58	24.2

* Means for all orchards combined, compared between years within a paired column, followed by a different letter are significantly different ($P < 0.05$).

collected in sweep net or limb jarring samples at orchard C and a total of 14 brown marmorated stink bugs were observed in visual samples between 25 May and 7 June with no other bugs documented for the remainder of the season (Figure 4(b)).

3.3. Trapping. In all four commercial apple plots sampled from August to November in 2010 and 2011, the number of adults ($t = 3.81$; df = 60.776; $P = 0.0003$) and nymphs ($t = 2.49$; df = 59.108; $P = 0.0155$) captured was significantly lower in 2011 (Figure 5(a)). On 8 September 2010, there was a significant shift in the ratio of adults:nymphs captured in traps at all orchards ($\chi^2 = 1762.3737$; df = 1; $P < 0.0001$). Prior to that date, significantly fewer adults were captured than nymphs at Orchard A ($\chi^2 = 21586.7131$; df = 1; $P < 0.0001$), C ($\chi^2 = 3410.2565$; df = 1; $P < 0.0001$), and D ($\chi^2 = 78.5714$; df = 1; $P < 0.0001$); however there was no difference between adult and nymph captures at orchard B ($\chi^2 = 1.5077$; df = 1; $P = 0.2195$). During the entire 2011 growing season, very few adults or nymphs were captured in traps deployed in apple and peach blocks; however, those that were captured were primarily recovered after July (Figure 5). In fact, 72% of all adult captures were recovered from traps on 29 September 2011.

3.4. Injury Assessments

3.4.1. Apple: 2010. In 2010, significantly more apples were injured on the plot exterior than in the interior at orchards A ($t = 2.18$; df = 18.836; $P = 0.0421$), B ($t = 4.48$; df = 15.964;

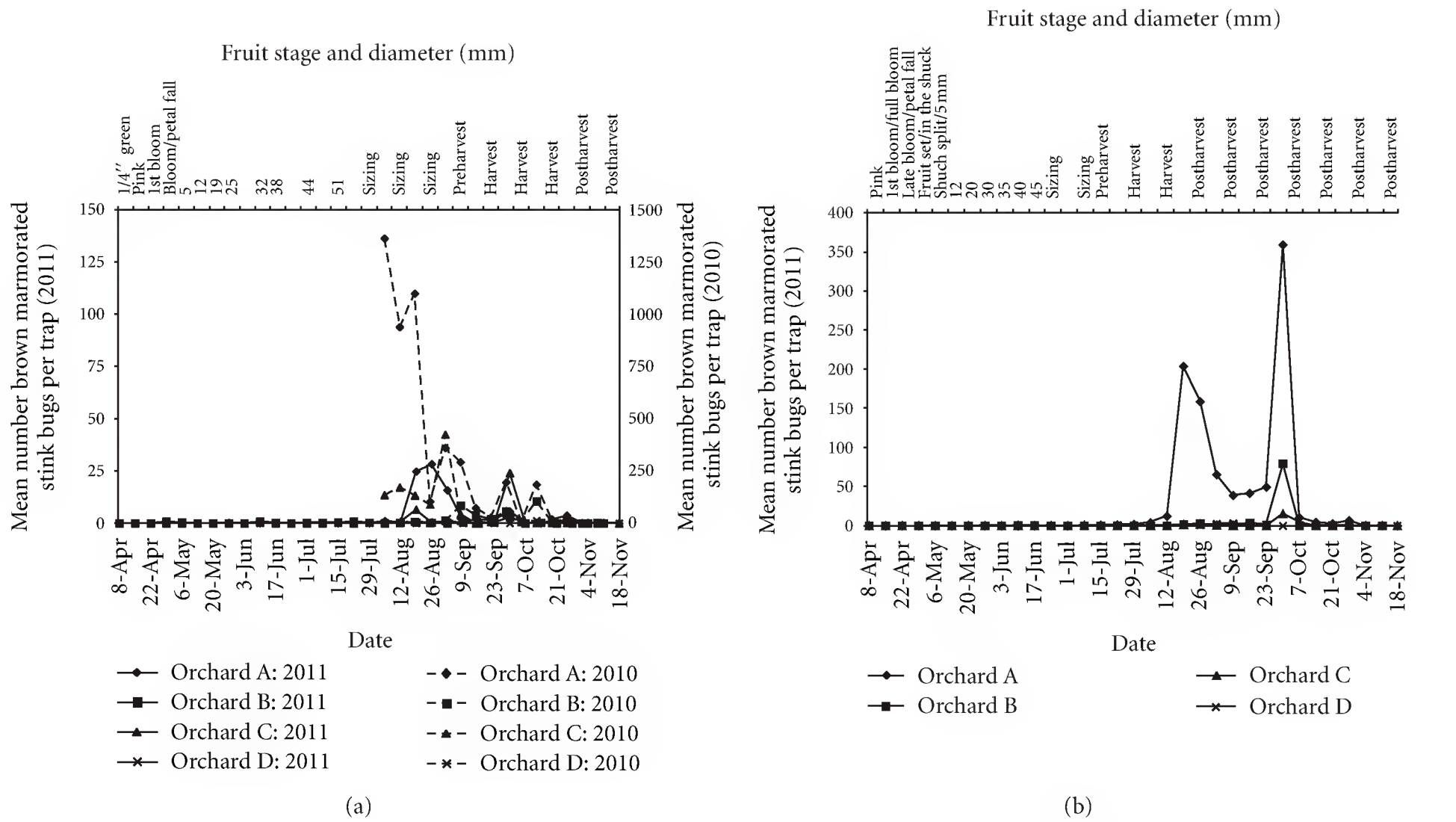


FIGURE 5: Mean number of brown marmrorated stink bug adults and nymphs captured per trap at orchards A-D in (a) apple in 2010 and 2011 and (b) peach plots in 2011.

TABLE 2: Total number of targeted brown marmorated stink bug insecticide applications, mean interval (d) ± SEM between insecticide applications, and mean A.I.M. score ± SEM and total A.I.M. score in peach plots from 2010 to 2011 at four commercial orchards.

Orchard	Number of targeted insecticide applications		Mean interval insecticide interval + SEM*		A.I.M. score Mean ± SEM*		Total	
	2010	2011	2010	2011	2010	2011	2010	2011
A	4	16	8.5 ± 1.0	6.7 ± 0.3	0.14 ± 0.03	0.30 ± 0.03	1.67	5.73
B	6	8	16.0 ± 1.9	15.7 ± 1.4	0.16 ± 0.03	0.25 ± 0.04	2.13	2.72
C	4	9	17.0 ± 3.2	14.1 ± 1.2	0.24 ± 0.06	0.30 ± 0.02	2.12	3.26
D	6	22	12.0 ± 2.6	4.5 ± 0.9	0.23 ± 0.05	0.18 ± 0.02	2.73	4.38
All Orchards	20	55	12.8 ± 1.2 a	8.1 ± 0.8 b	0.18 ± 0.02 a	0.25 ± 0.01 b	8.65	16.09

*Means for all orchards combined, compared between years within a paired column, followed by a different letter are significantly different ($P < 0.05$).

$P = 0.0004$), and C ($t = 2.64$; df = 9.4638; $P = 0.0258$); however, there was no statistical difference at orchard D ($t = 0.87$; df = 14.901; $P = 0.4007$) (Figure 6(a)). No differences in the number of injury sites were observed between apples on the exterior or interior of plots for any orchard (A: $t = 1.20$; df = 19.761; $P = 0.2452$, B: $t = 1.02$; df = 15.96; $P = 0.3211$, C: $t = 1.38$; df = 11.963; $P = 0.1923$, D: $t = -0.22$; df = 14.474; $P = .8273$) (Figure 6(b)).

3.4.2. Peach: 2010. Fruit sampling in peach started relatively close to harvest in 2010, so few samples were available for comparison and thus only data summaries were performed. The percent injury and number of injury sites recorded at harvest did not vary greatly from injury recorded at the first sample. Orchard A and B had more injured fruit on the exterior; however the reverse was true at orchard D. There was no interior peach sample at orchard C due to the size and layout of the plot. At the three orchards that had both exterior and interior peach samples, the number of injury sites per injured fruit was higher on the exterior of the plots (Figures 7(a) and 7(b)).

3.4.3. Apple: 2011. In 2011, significantly more apples were injured on the plot exterior than in the interior at orchard A ($t = 2.89$; df = 10.473; $P = 0.0153$), C ($t = 6.43$; df = 24; $P < 0.0001$), and D ($t = 2.61$; df = 18.507; $P = 0.0174$); however, there was no statistical difference at orchard B ($t = 1.75$; df = 6.8897; $P = 0.1235$) (Figure 8(a)). Only orchard C ($t = 2.53$; df = 21.895; $P = 0.0189$) had significantly more injury sites per injured apple on the plot exterior than on the interior (Figure 8(b)).

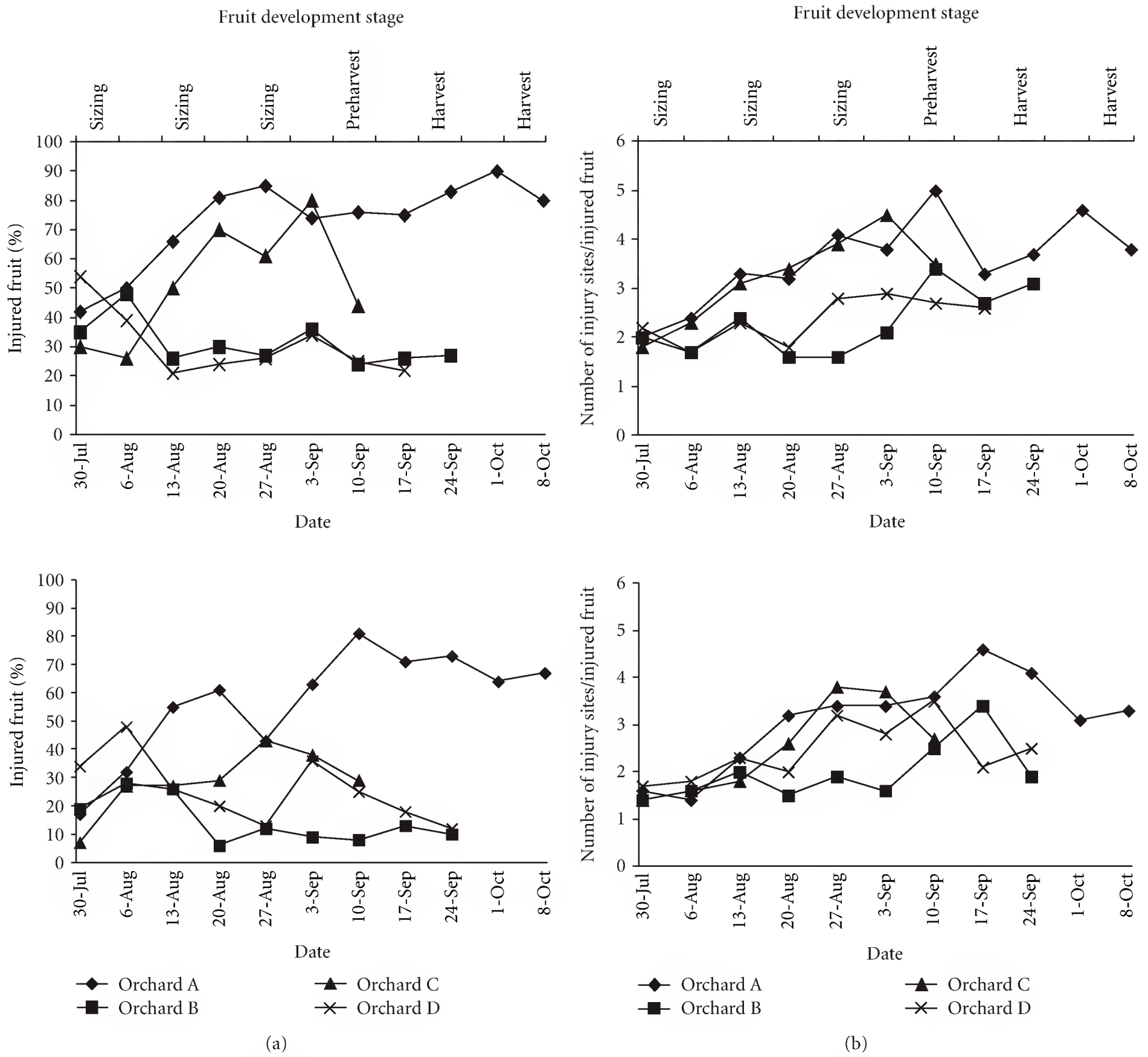


Figure 6: (a) Percent injured apples in 2010 at orchards A–D from the plot exterior (top) and interior (bottom) and (b) the number of injury sites per injured fruit in 2010 at orchards A-D in the plot exterior (top) and interior (bottom).

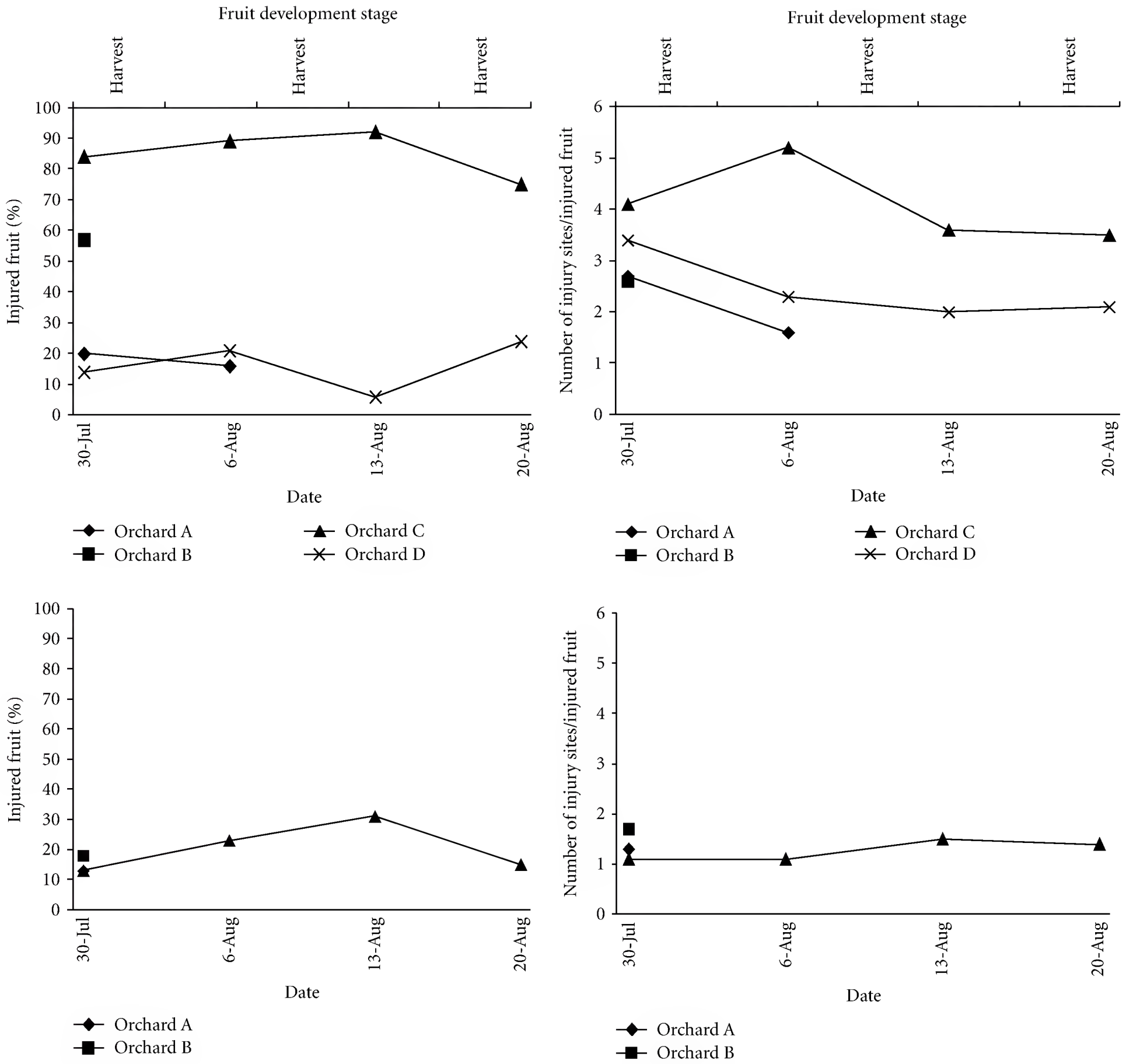
3.4.4. Peach: 2011. In the peach plots, orchard B ($t = 2.13$; df = 17; $P = 0.0477$) and D ($t = 3.34$; df = 17.451; $P = 0.0038$) had significantly more injured fruit on the exterior than in the interior, whereas there was no difference at orchard A ($t = -0.50$; df = 16.598; $P = 0.6230$) or C ($t = -0.46$; df = 13.548; $P = 0.6548$) (Figure 9(a)). Only orchard B ($t = 4.14$; df = 4; $P = 0.0143$) had significantly more injury sites per injured peach on the exterior of the plot than the interior; there was no difference at the other orchards (Figure 9).

3.4.5. Apple: 2010 versus 2011. Finally, significantly more apples were injured from 30 July through harvest in total in 2010 than 2011 at each orchard (A: $t = 13.25$; df = 40; $P < 0.0001$, B: $t = 5.03$; df = 33; $P < 0.0001$, C: $t = 5.32$; df = 38; $P < 0.0001$, D: $t = 5.69$; df = 40; $P < 0.0001$). Likewise, the total number of injury sites per injured fruit was significantly greater in 2010 than 2011 at orchard A ($t = 6.51$; df = 34.78; $P < 0.0001$), C ($t = 2.45$; df = 25.765; $P = 0.0212$), and D ($t = 4.83$; df = 32.231; $P < 0.0001$), but not at orchard B ($t = 1.74$; df = 26.376; $P = 0.0940$).

3.4.6. Peach: 2010 versus 2011. No statistical comparisons were performed due to minimal peach samples collected in orchards in 2010. However, injury was generally higher in both exterior and interior samples in 2010 compared with 2011 at harvest.

4. Discussion

Brown marmorated stink bug has been documented as utilizing apple as a host in Japan [26] and the United States



(a) (b)

FIGURE 7: (a) Percent injured peaches in 2010 at orchards A–D from the plot exterior (top) and interior (bottom) and (b) the number of injury sites per injured fruit in 2010 at orchards A–D in the plot exterior (top) and interior (bottom).

[6]. Nielsen and Hamilton [7] found that based on a caging study, injury was significantly greater during the late-season compared with petal fall or mid-season. In our studies, we found that natural populations of brown marmorated stink bug in commercial apple blocks will feed on fruit throughout the season, but like native stink bug species [25], feeding injury that occurs in the early season results in a small feeding puncture in the fruit skin and nominal injury to the flesh, while injury inflicted 6-8 weeks after petal fall until harvest results in indented depressions on the surface of the fruit with corky flesh beneath [25, 27]. However, like native stink bugs [25, 27] injury symptoms may take several weeks to manifest completely (S. Joseph, personal communication). Native stink bugs found in mid-Atlantic tree fruit orchards in the United States include *Euschistus servus*, *E. tristigmus*, and *C. hilaris* predominantly [11]. These species will feed on many cultivars of apples, though higher injury rates have been recorded, in one study, on "Braeburn," "Jonica," "Jonagold," "Starkspur Dixiered," "Granny Smith" and "Stayman" [28]. However in our study, no direct comparisons of cultivar susceptibility were conducted. Injury patterns within apple blocks indicate the brown marmorated stink bug is a perimeter-driven threat. Indeed in 2010 and 2011, injury was usually significantly greater at the exterior of orchard blocks

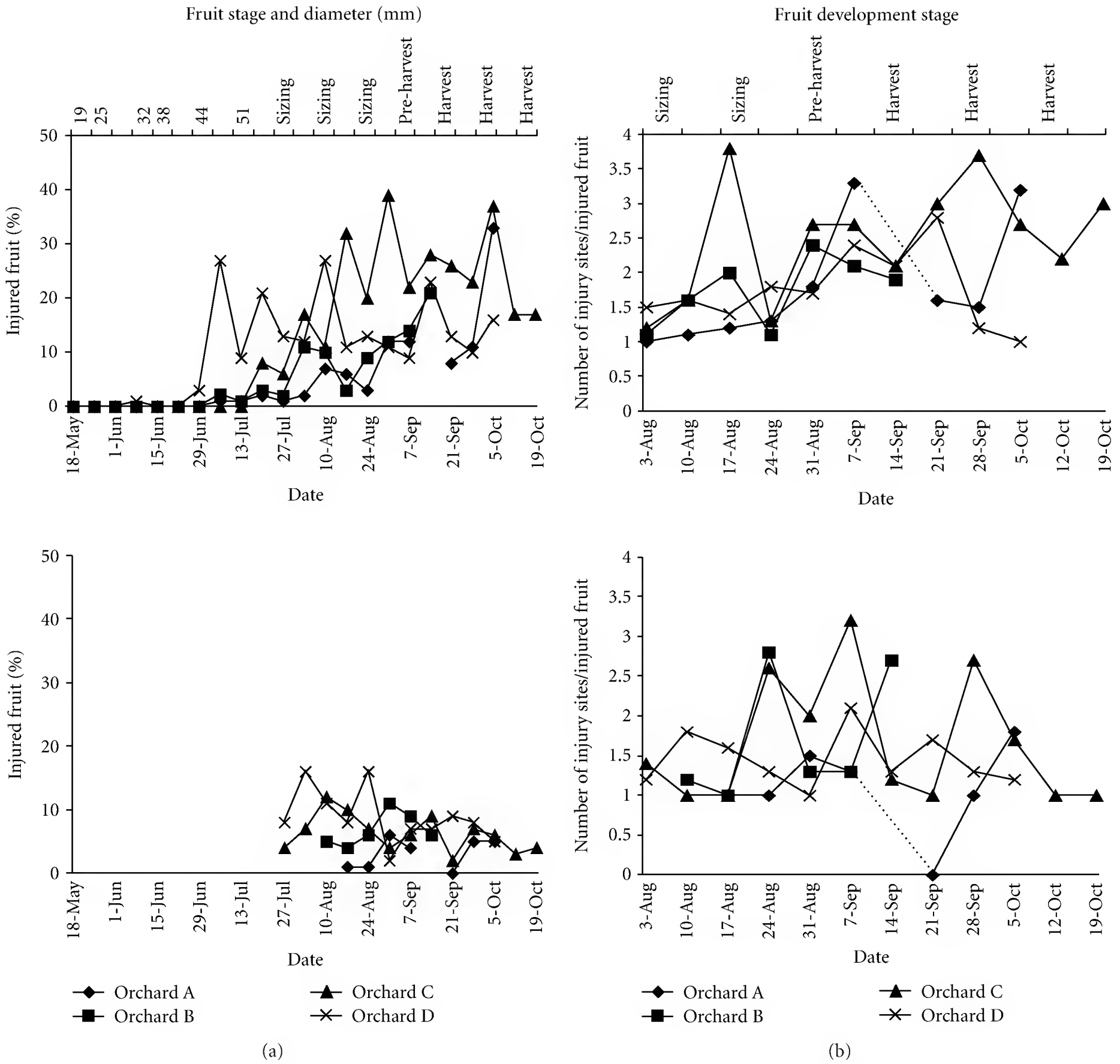


FIGURE 8: (a) Percent injured apples in 2011 at orchards A–D from the plot exterior (top) and interior (bottom) and (b) the number of injury sites per injured fruit in 2011 at orchards A–D in the plot exterior (top) and interior (bottom). Note: the dotted lines in 8B represent missing data on 14 September.

relative to the interior suggesting that adults, emigrating from overwintering sites in the early season and from wood lots or cultivated hosts such as corn and soybean later in the season, constantly invade orchards. Similar patterns of movement have been observed for native stink bug species in other cropping systems [29, 30].

Peach is also an excellent host for brown marmorated stink bug. In cage studies, brown marmorated stink bug caused the greatest injury during the late season [7]. In our studies, natural populations of adults have proven to be extremely damaging in commercial peach orchards in the early season soon after fruit set. In 2011, large numbers of adults moving from overwintering sites began to target the developing peach fruit by 1 June (~30 mm diameter fruit); two orchards had already recorded over 20% damage. In 2010, early-season feeding by adults led to devastating injury to peach growers in many mid-Atlantic states [8]. Unlike apple injury, peach symptoms appeared to manifest very quickly after feeding, within several days. Typically injury inflicted by native stink bugs results in cat-facing and gummosis [31], while early season brown marmorated stink bug injury, though resulting in gummosis, often results in dead pockets of tissue deep in the flesh of the fruit that are not obvious on the surface as the fruit matures. While native stink bugs are capable of inflicting this type of injury as well, it has proven far more prevalent from brown marmorated stink bug. Damage in commercial peach blocks was significantly greater in the exterior compared with the

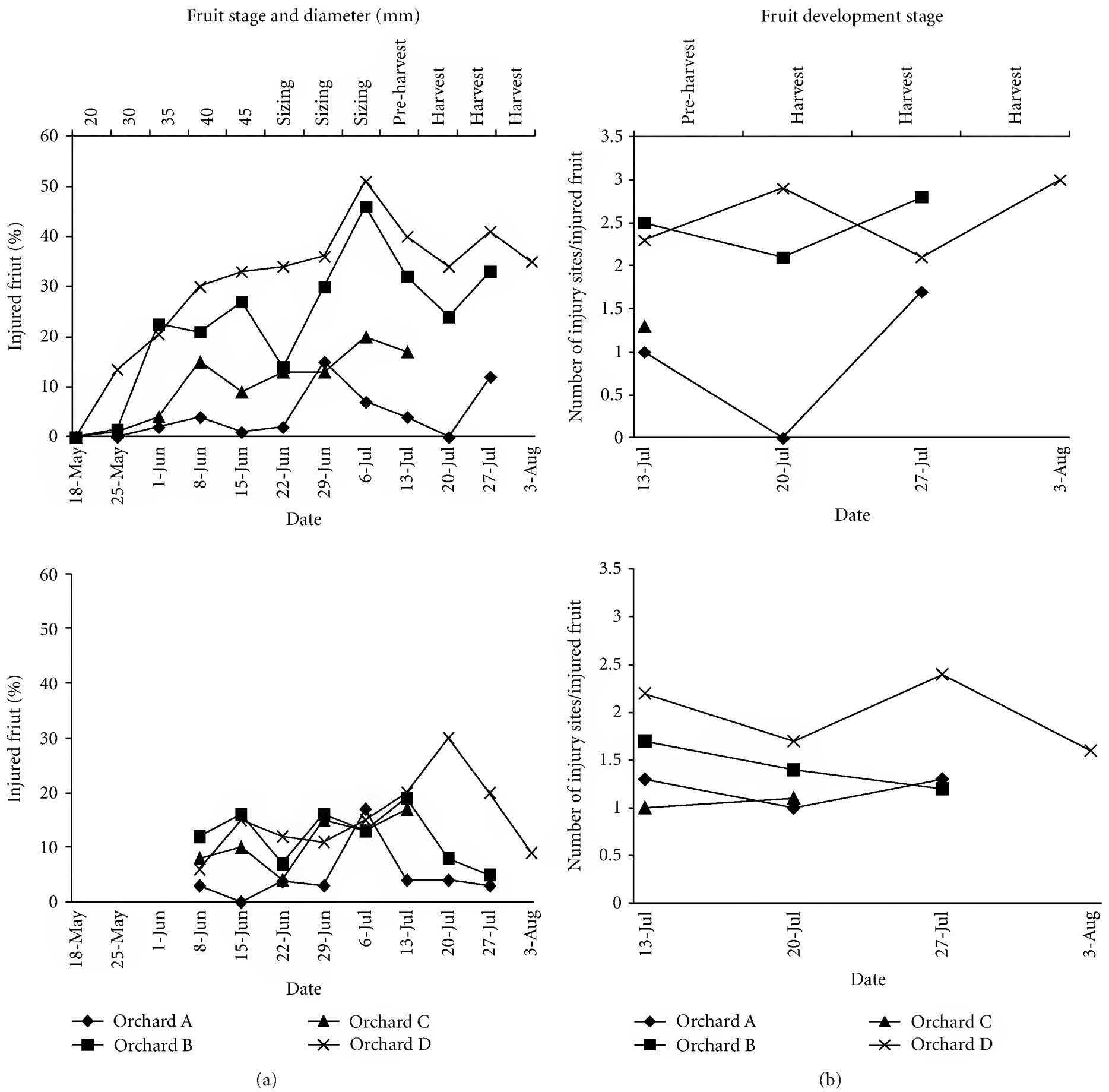


FIGURE 9: (a) Percent injured peaches in 2011 at orchards A–D from the plot exterior (top) and interior (bottom) and (b) the number of injury sites per injured fruit in 2011 at orchards A–D in the plot exterior (top) and interior (bottom).

interior, indicating adults were immigrating into blocks from the outside as was found for apple.

Based on the presence of economic injury, peach fruit is vulnerable soon after fruit set and this vulnerability continues to harvest. By contrast, economic injury to apple generally begins 6–8 weeks after petal fall and can continue until harvest (Figure 8). However, because reproduction can occur in commercial orchards, mitigating treatments must be undertaken early to prevent the threat of nymphal populations contributing to overall injury.

In apple and peach orchard blocks, growers changed their management programs tremendously from 2010 to 2011. In 2010, growers either did not target brown marmorated stink bug specifically or used materials recommended for native stink bugs, particularly pyrethroids. In peach orchards in the southeastern United States, where cat-facing bugs including native stink bugs and tarnished plant bug, *Lygus lineolaris* Palisot de Beauvois, are considered key pests, control is typically achieved using pyrethroids [32]. Unfortunately, many of these same materials did not provide adequate control of brown marmorated stink bug in 2010, as documented in a field trial in which over 25% of moribund bugs exposed to cyfluthrin in treated apple canopies recovered to an actively foraging state [16]. Furthermore, in laboratory trials, treatments of beta-cyfluthrin, lambda-cyhalothrin, zeta-cypermethrin, cyfluthrin, and esfenvalerate resulted in high initial knockdown of adults, but also high levels of recovery [15].

Thus, in 2011, growers relied on materials such as endosulfan, methomyl, permethrin, fenpropathrin, dinotefuran, clothianidin, and thiamethoxam. All of these materials not only demonstrated high levels of immediate knockdown, but also very little recovery [15]. In general, the mean AIM score showed that a more intense insecticide program was implemented in 2011 in response to the threat posed by brown marmorated stink bug. Likewise, the total AIM score illustrates that growers used more of these targeted spray applications in 2011 than in 2010. In addition, growers shortened the intervals between insecticide applications from 2010 to 2011. For apple plots in 2010, growers sprayed at approximately 2-wk intervals but cut that to a 1-wk interval in 2011. Similarly in peach plots in 2010 growers, treated at approximately 13-d intervals but shortened that to 8-d intervals in 2011. These factors likely contributed to both the decrease in overall percent injury and lower trap captures. However, the sustainability of this type of program financially and ecologically is not feasible and growers will certainly need to implement a sensitive and reliable monitoring program for future seasons.

Monitoring tools can be used to effectively assess presence, abundance, and seasonal activity of a pest species, allowing growers to make informed management decisions. In 2010 and 2011, we evaluated the use of black pyramid traps baited with methyl-(2*E*,4*E*,6*Z*)-decatrienoate as a monitoring tool for brown marmorated stink bug populations. This trap and lure combination had been previously shown effective at capturing large numbers of adults and nymphs of brown marmorated stink bug [21]. Significantly more adults were captured in traps in 2010 compared with 2011. This pattern likely reflects four key considerations. First, in 2010, as stated above, growers were not specifically targeting brown marmorated stink bug with insecticide applications, allowing adults to reproduce and populations to build within the orchards. Second, weather patterns were quite different between years. In 2010, a second generation of adults had completed development by 13 September [21], whereas in 2011, development was not complete until 11 October (Leskey, unpublished data). Based on developmental rates developed by Nielsen et al. [33], degree day accumulations required for completion of the second generation were much slower in 2011, possibly leading to much smaller populations observed and subsequently trapped. Third, a different trap top was used in 2011 than in 2010. This trap top has subsequently been reported to be less effective in capturing and/or retaining brown marmorated stink bugs. Finally, differences in overall lure effectiveness may exist between 2010 and 2011 based on overall trap captures and release rates [34] contributing to lower captures in 2011.

However, more problematic is the fact that very few adults were captured in traps throughout the growing season in 2011. It is true that growers instituted season-long management programs in 2011 against brown marmorated stink bug; however, injury rates early in the season indicate that adults were surely present. Funayama [35] captured brown marmorated stink bug adults using traps baited with methyl-(2*E*,4*E*,6*Z*)-decatrieonate early in the season during outbreak years. However, our traps did not recover adults, even remotely reflective of observed populations, until mid-August in apple. Indeed, greatest trap captures actually occurred in peach orchards after the fruit was harvested. Thus, other monitoring techniques were evaluated. As evaluated for native stink bugs in the mid-Atlantic [11], we tested the use of sweep nets and limb jarring samples in commercial orchards season-long in 2011. Almost no bugs were recovered from these sampling techniques indicating that they did not adequately reflect the presence, size, or activity of populations. Timed visual counts also were conducted and did result in greater numbers of bugs being observed, but again the numbers were too low to adequately reflect population density or activity. It is likely that observed behavioral attributes of brown marmorated stink bug are not compatible with these sampling techniques. For example, brown marmorated stink bug has a tendency to be found high in the tree (Short, personal observation) lending to difficulty in obtaining individuals from limb jarring samples taken at head-height or below. Adults have not been observed feeding on many broad-leaf weeds based on host surveys conducted to date [5], which could lead to fewer captures in sweep nets. Likewise, diurnal patterns are not well understood. Certainly, adults and nymphs have been observed actively feeding and mating at night by numerous researchers.

Although cross attraction to methyl-(2*E*,4*E*,6*Z*)-decatrieonate has been observed for brown marmorated stink bug [21] as well as other species including *C. hilaris* [36], it is not attractive to brown marmorated stink bug adults season-long. Thus, identification of the aggregation pheromone of this species could provide a better tool to use with baited traps. Additionally, brown marmorated stink bug adults do respond to visible light (Leskey, unpublished data) and to ultraviolet light as they have been captured in black light traps [6]. Perhaps, creating a trap with optimized olfactory and visual stimuli including the true aggregation pheromone and specific wavelengths of light could provide a much more sensitive monitoring tool that is attractive season-long and can be used to develop treatment thresholds for this invasive species. In the interim, however, it is likely that growers still will need to continue with aggressive management programs aimed at this invasive species in order to mitigate economic injury and successfully grow tree fruit in regions where it is well established.

Acknowledgments

The authors thank J. Cullum, T. Hancock, R. Posa, C. Scorza, and S. Wiles for excellent technical support and the commercial growers in West Virginia and Maryland who cooperated with the studies. This paper was supported by a specific cooperative agreement with USDA-APHIS and by USDA-NIFA SCRI no. 2011-51181-30937.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 942737, 6 pages
doi:10.1155/2012/942737

Research Article

Foraging Activity and Trophic Spectrum of Red Ant *Pogonomyrmex barbatus* Smith, 1858, in Productivity-Contrasted Microenvironments

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Received 2 February 2012; Revised 17 May 2012; Accepted 18 May 2012

Academic Editor: James Charles Nieh

Foraging strategies can be influenced by many factors such as abundance, availability, and toxicity of the resources. In arid zones, the distribution and productivity of plants also act as additional factors that affect foraging strategies. Twenty colonies of *Pogonomyrmex barbatus* ants were studied in an arid zone of central Mexico to evaluate the trophic niche breadth in two sites with contrasting productivities in terms of their diversity and amount of resources during two seasons. The results suggest that when the resources are abundant as in the rainy season, the trophic niche breadth is reduced in sites with high productivity and, in the same sites, the trophic niche breadth increases when the resources are limited as in the dry season. In contrast, the trophic niche breadth is similar in both conditions of resource availability (i.e., rainy and dry seasons) at sites with low productivity. During the dry season, populations of *P. barbatus* showed a similar foraging behavior in sites with high and low productivity. Thus, the particular characteristics of a site can significantly affect the foraging strategies of the ants in those environments.

1. Introduction

Food options for organisms are often influenced by several resource characteristics such as availability, distribution, toxins content, palatability, and acceptance, and by the behavior and biology of the organisms, including life cycle, tolerance to environment changes, and feeding habits restrictions [1, 2]. Gordon [3] enumerated the mechanisms behind diet modification in ants in relation to changes in the foraging area: an increase in territory results in higher levels of resources, cost in territory defense, risk of predation, and energy used for gathering and transporting the resources [1, 3]. Other factors that may affect the availability or not of the resources in ant foraging areas include localization, because physical conditions can be very important in the colony development; food availability; intensity of biotic interactions such as inter- and intraspecific competition [4, 5].

According to the optimal foraging theory (OFT), organism must develop cost-effective strategies to obtain more resources and energy by using mechanisms favored by natural selection, resulting in a positive impact on the species fitness [2]. In the case of ants, which are highly diverse and abundant [6], foraging mechanisms involve three general patterns: hunting (including predation and granivory), rewards (e.g., exploitation of extrafloral nectaries, elaiosomes, and homopteran secretions), and defense for discovered resources [7]. Depending on the ant species,

cognitive plasticity (learning) and the use of visual signals are important foraging mechanisms, as observed in *Ectatomma ruidum* (Roger, 1860) and *E. tuberculatum* (Olivier, 1792) [8], and in *Pogonomyrmex* sp., whose learning is related to site fidelity [7].

In arid ecosystems, primary production occurs in pulses attributable to rainy seasonal patterns [9] that produce a high environmental heterogeneity as result of the unequal distribution of humidity in time and space [10]. These seasonal patterns are relevant because little modifications in the ecosystem's components, such as precipitation, can influence variations in other elements, thus generating various microhabitats with different productivities, composition and abundance of primary producers, primary consumers, and predators [11–13].

Resource abundance is an indicator of productivity in terms of energy availability. Organisms modify their feeding behavior in relation to food availability in the habitat. In the case of resource scarcity, several important coincidences in the diet of species have been recorded, increasing the competition for food [14]. In contrast, feeding specialization occurs under conditions of food abundance [2, 15].

Although the arid zones have been regarded as sites with low ant diversity, recent investigations have revealed a remarkably high diversity and abundance [6, 16, 17], together with highly variable interactions and trophic habits, as influenced by time. These reports indicate that ants play relevant roles in various ecosystem processes, including nutrient recycling and redistribution of resources [18].

The aim of the this study was to examine the foraging strategies of *Pogonomyrmex barbatus* (Smith, 1858) in relation to habitat productivity by attempting to answer the following question: how does the trophic spectrum of *P. barbatus* influence habitat productivity in a semiarid zone? We assumed that productivity would be directly related to the diversity and abundance of food [11, 19, 20], allowing a direct relationship between productivity and resources diversity and abundance. Our hypothesis was that, under relatively high productivity, the feeding habits of *P. barbatus* would be more specific, whereas in habitats with relatively low productivity, the feeding habits would follow a generalist behavior. High and low productivities in a habitat are defined in this study as a function of the plant species richness and food abundance.

2. Materials and Methods

2.1. Study Sites. The study was performed at the "Helia Bravo" Botanical Garden (18°27′30″N, 97°24′50″W) at 1678 m a.s.l., located in the Zapotitlán Salinas Valley, into the physiographic region of Tehuacán-Cuicatlán, in Puebla and Oaxaca States, Mexico. The weather is generally dry with a rainy period from May to October each year and 400 mm of annual average precipitation, and a dry season from November to April. The annual average temperature ranges from 18 to 22°C. The dominant vegetation consists of xerophytic shrub, as reported by Rzedowski [21], with physiognomic variations related to the local environmental conditions, resulting in different vegetation types [22].

Two sites in the Botanical Garden, each with contrasting productivities based on plant cover, species richness, and productivity, were selected. The first site was named Jardín (18°19.78′N, 97°27.45′W) and showed the highest values of plant cover (116.36%) and species richness ($S = 25$), when compared to those of the second site named Llano (18°19.54′N, 97°27.26′W), which is located in a zone with high erosion (plant cover = 45.54%; species richness = 16). The distance between the sites, estimated with a Garmin 60 C GPS, was 600 m in a linear direction, although a hill was located between the two sites. Species similarity between the two plant communities was estimated as 12.2% by using the Renkonen similarity index. The availability of resources at the Jardín site, according with a preliminary study by Guzmán-Mendoza [23] was 2,252 seeds of different species, 175 remains of vegetal material (branches, leaves, and parenchymal tissue of leguminous pods), and 1,379 objects of animal material (insects, exuviae, spiders, and caterpillars) per 600 m^2. At the Llano site, the available resources included 12,760 seeds, 470 plant material remains, and 1,004 animal materials per 600 m^2. The amount of resources differed in relation to the site; thus, the Llano site possessed a greater variety of resources (Jardín: $\chi^2_{0.05,12} = 634.46$, $P < 0.0001$; Llano $\chi^2_{0.05,12} = 5663.86$, $P < 0.0001$), and season (rainy season: $\chi^2_{0.05,12} = 1141.14$, $P < 0.0001$; dry season: $\chi^2_{0.05,12} = 4805.67$, $P < 0.0001$). The composition of resources (i.e., seeds types and animal composition) was similar in both sites and seasons.

2.2. Foraging Activity of Pogonomyrmex Barbatus. To establish the intensity of foraging activity, the number of ants engaged in searching and gathering resources for an approximated duration of 8 minutes was counted. In each site (Jardín and Llano) were studied ten colonies for a total of 20 colonies studied in the area. In each observation, the colony disk was divided into four quadrants with directions NE, SE, SW, NW, and each quadrant was observed for 2 minutes. The ants leaving or joining the colony was recorded for each quadrant, counting only those that crossed the disk border. All data were analyzed using two-way analysis of variance (ANOVA) to compare the number of ants engaged in foraging between sites and seasons. Significant differences were tested using the least significant differences (LSDs) multiple comparison test [24].

Trophic niche breadth was estimated from the recorded number of ants returning to the nest with objects in their mandibles. Observations were performed for approximately 20 minutes. The objects carried by the ant using their mandibles were removed using entomological forceps and were assigned to one of the categories previously mentioned. To measure niche breadth, Levins index [25] was used to estimate the width, which was used as a measure of distribution of individuals uniformity among resources. The index value is highest when individuals are observed in all resources, and the minimum value is observed when the individuals are present in only one resource [25]. We estimated the diversity

Table 1: Results of two-way ANOVA test for the effect of site, season and interaction on the foragingintensity of *Pogonomyrmex barbatus* at the "Helia Bravo" Botanical Garden, Puebla, Mexico. Significant level $\alpha = 0.05$.

Variation source	Square sum	F value and probability
Sites	3971.0	$F_{0.05(1)1} = 1.135$; $P = 0.293$
Seasons	68967.4	$F_{0.05(1)1} = 19.719$; $P = 0.001$
Site ∗ season	18409.1	$F_{0.05(1)1} = 5.263$; $P = 0.027$

Table 2: Results of multiple comparison LSD tests for ant foraging during dry and rainy season in two sites, Jardín and Llano. $\alpha = 0.05$. LlR: Llano rainy; LlD: Llano dry; JR: Jardín rainy; JD: Jardín dry. Distinct letters indicate significant differences.

Site-season	Difference average	Probability	Confidence intervals (95%)	
LlD_a				
LlR	120.09	<0.05	171.05	69.12
JD_a	21.90	>0.05	72.87	29.05
JR	60.18	<0.05	111.14	9.21
LlR				
JD	98.18	<0.05	47.21	149.14
JR	59.90	<0.05	8.94	110.87
JD_b				
JR_b	38.27	>0.05	89.23	12.69

of resources by using the Shannon index and compared the results obtained in both communities [25]. The comparisons were made between sites and seasons. Data analyses were performed using SPSS 12.0 software (SPSS INC. 2003 SPSS for Windows rel. 12.0, Chicago IL, USA).

3. Results

3.1. Foraging Intensity. There were differences in the number of ants engaged in foraging in both sites and seasons. During the rainy season, the Llano colonies showed a higher number of foraging ants (average ± se = 53.5 ± 22.73) than the Jardín colonies (20.65 ± 18.96). During the dry season, the observed pattern was reversed: the Jardín colonies were more active (15.07 ± 9.73) than the Llano colonies (6.59 ± 7.17; Table 1). The results of ANOVA test showed significant differences in the site and season, and the LSD multiple-comparison test, LSD reveled that ants were more active during the rainy season, regardless of site (Table 2). The lowest values in activity for both sites (Figure 1) were recorded during the dry season. However, the foraging activity in Jardín was similar during both seasons (MD = 38.2, $P = 0.137$). Nevertheless, in Llano, the season significantly influenced the foraging ant activity (Table 2).

3.2. Trophic Niche Breadth. Similar to the study by Guzmán-Mendoza [23], the heterogeneity of available resources was similar in both sites ($t_{0.05,17.97} = 0.66$, $P = 0.52$), despite the greater abundance of seeds recorded in Llano. The

Table 3: Trophic niche breadth of *Pogonomyrmex barbatus* at two semiarid sites with contrasting productivities during the rainy and dry season. JR: Jardín rainy; JD: Jardín dry; LlR: Llano rainy; LlD: Llano dry.

Site-season	Levins standardized index	Shannon diversity index	t value and probability
JR	0.31	0.95	
LlR	0.17	0.81	$t_{0.05(44)} = 4.96$, $P < 0.0001$
JD	0.16	0.76	
LlD	0.14	0.73	$t_{0.05(34)} = 0.713$, $P = 0.48$

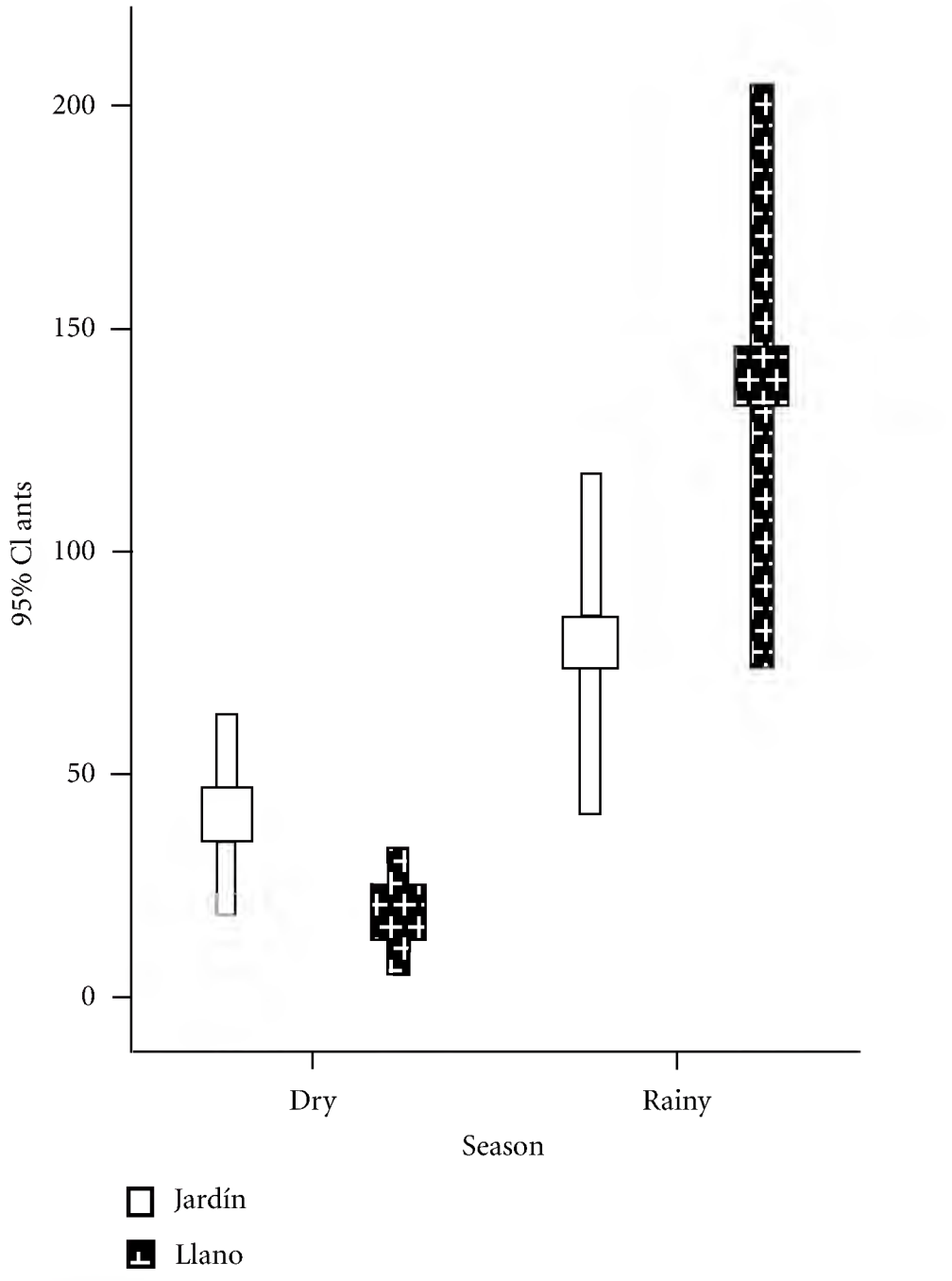

Figure 1: Average foraging activities of active ants at two sites at the Zapotitlán Valley, Puebla, Mexico, with contrasting productivities during two seasons.

comparison of trophic niche breadth showed that the Jardín colonies exhibited a stronger generalist approach than the Llano colonies ($t_{0.05,44} = 4.96$, $P < 0.0001$, Table 3). However, both sites showed that more seeds were used as a major resource on the basis of the observed number of ants physically carrying this specific resource (Figure 2).

Despite the variety of resources available to the colonies, the ants of Jardín mainly foraged on seeds, arthropods, floral structures, leaves and excreta, whereas the Llano ants

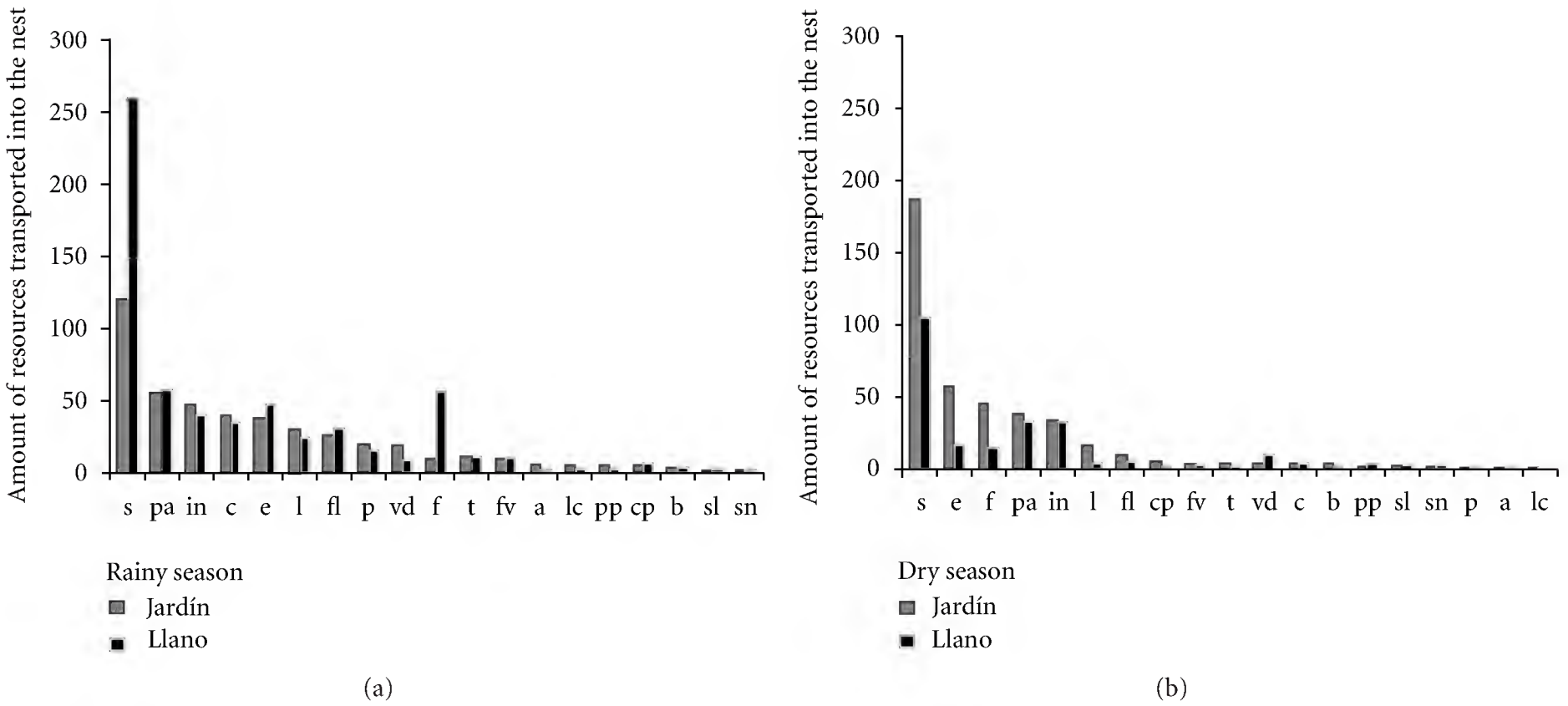

FIGURE 2: Resources removed from the ant mandibles during the rainy and dry season in two contrasting sites at the "Helia Bravo" Botanical Garden at Zapotitlán de las Salinas, Puebla, Mexico. a: algae; b: buds; c: capitate; cp: caterpillars; e: excretes; f: fruits; fl: flowers; fv: fleshy vegetal tissue; in: insects; l: leaves; lc: lichen; p: peels; pa: pieces of arthropods; pp: pupae; s: seeds; sl: soil; sn: snails; t: twigs; vd: vegetal debris.

were more actively focused on seeds, fruits, arthropods, and excreta.

During the dry season, the amount of resources differed at both sites. More resources and heterogeneity were recorded at Jardín than at Llano ($t_{0.05,17.97} = 44.43$, $P < 0.0001$). The comparison between trophic niches breadth values showed no significant differences during this season ($t_{0.05,34} = 0.713$, $P = 0.48$, Table 3).

4. Discussion

Results from counting the number of foraging ants in relation to trophic niche breadth suggest a direct and positive relationship between both variables. A high number of foraging ants indicated a wider range of choices diet, as observed in the Llano site during both rainy and the dry seasons. When resources were limited, some foraging ants invested less time searching for resources, and instead, focused on the most common resources in the area, such as seeds, insect fragments, excreta, and fruits, as shown in Figure 2. This pattern is related to the season, humidity, and temperature conditions. However, other ant species can search for complementary food sources to increase their trophic spectrum and foraging efficiency [7]. On the basis of these results, our study does not completely agree with the optimal foraging theory (OFT) that predicts wide-range diets in low productive environments, as compared to limited-range diets in high productive environments [2]. Although that theory has been tested in different cases [14, 26, 27], evidence for granivory systems are limited [28], and it seems that the behavioral peculiarities of ants related to patterns of foraging for resources, make them to perform somehow away from the predictions of OFT [29].

The number of foraging ants is related to trophic niche breadth, but the patterns of increase or decrease in the number of foraging ants depend on the environmental conditions related to the season, as shown by the recorded humidity and temperature values. Seasonality is an important factor for niche breadth of *P. barbatus* at the two studied sites, and attributable to the availability of resources and time of foraging, which are directly related to the humidity and temperature of the soil surface. In other arid zones, ants belonging to other species of *Pogonomyrmex* genus showed differences in their foraging habits in response to an environmental gradient; some of them preferred the highest temperatures during the day, whereas others showed peak foraging activity during the coldest hours of day [27]. At the Jardín site, the changes number of foraging ants were attributable to abundance of resources, whereas the diversity of resources at the Llano site showed a modified niche breadth; here, a wider trophic niche was observed with higher diversity levels and more forager ants. Thus, ants at the Jardín site under conditions of high abundance and diversity of resources reduced their trophic niche breadth and activity and were more generalists to a greater extent when the diversity of resources was limited. These results suggest that trophic niche breadth is not influenced by resource abundance alone, contrary to the assumption of the OFT [2].

Although differential abundance of resources can modify the niche breadth, as reported in other organisms such as fishes [14], tadpoles [30], and several butterflies species, and other animals present at a site that was in its first stages of succession after perturbation events and in which food was limited [15, 31], these conditions promote species superposition of diets and strong competition for food. Thus, the abundance and diversity of resources can play an

important role in the establishment of variations in trophic niches. On the basis of the results of this study, it is possible to identify particularities in resource use according to inherent features of each site.

The results obtained in this study may increase knowledge on the feeding scheme of ants, which are important species because of their abundance and diversity but have been poorly studied in terms of their feeding relationships [32]. Nevertheless, it is necessary to conduct more observations and field experiments to quantify the influence of other parameters on ant diet, such as age, species diversity, and predation, to understand the role of the ants in the food web of arid ecosystems [32, 33].

Acknowledgments

The authors would like to thank CONACyT for the postgraduate scholarship awarded to R. Guzmán-Mendoza, the Zapotitlán de las Salinas habitants, Pedro Miranda, and Agueda Vásquez-Sosa by their help during the field work.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 585014, 10 pages
doi:10.1155/2012/585014

Research Article

Cue-Mediated Recruitment in a Swarm-Founding Wasp: Successful Foragers Induce Nestmates to Search off Nest for a Scented Carbohydrate Resource

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Received 26 February 2012; Revised 4 May 2012; Accepted 6 May 2012

Academic Editor: James Charles Nieh

The ability of social insect colonies to recruit nestmates to profitable resources increases colony-wide foraging efficiency by providing individuals with information that narrows their search for resources. Here we ask if for the Neotropical swarm-founding wasp *Polybia occidentalis* naïve nestmates are able to use food-scent cues from rich carbohydrate resources brought to the nest by successful foragers to orient to off nest resources. Foragers were allowed to freely visit a training dish containing a scented sucrose solution. At a second location, in a different direction from the nest, two sucrose-filled dishes were offered, one with the training scent and one with an alternate scent. Naïve foragers preferentially chose the training scent over the alternate scent, indicating that natural rates of resource inflow to the nest are sufficient to induce nestmates to forage at resources with a specific scent. Naïve foragers did not forage more often at the location at which the active foragers were foraging, an indication that directional information is not communicated in this species. The total number of foraging trips made by a colony's foragers was not determined by the size of the foraging force, but rather by the average individual foraging rate for the colony.

1. Introduction

Recruitment in social insects has been variously defined as communication that brings individuals to where work is needed [1], "(a)ny behavior that results in an increase in the number of individuals at a particular place" [2, page 115], and "the local increase of workers cooperating at a particular place" [3, page 29]. Recruitment to food enables foragers to exploit patchily distributed resources more efficiently than is possible by independent searching, because it allows them to make use of information that reduces the uncertainty of finding the resource [4, 5]. For species that store excess food reserves in the nest, recruitment allows the colony to more quickly and fully exploit bonanzas that temporarily exceed immediate demand [6, 7].

Although recruitment is often narrowly defined as exclusively signal mediated [8–10], it may also be mediated by social cues [2, 3, 6, 7, 11–14]. Thus, one may distinguish between "signal-based recruitment" and "cue-based recruitment." The waggle dance of the honey bee and pheromone trails of ants are examples of the former, whereas the social wasps' use of food odors brought to the nest and local enhancement at feeding sites are examples of cue-based recruitment to food resources [2, 15]. Although most ant and many bee species have evolved signals used in recruitment to food, they no doubt also make use of social cues [3, 15–19]. In contrast, the social wasps appear to lack signal-based recruitment to food and to rely exclusively on cue-based mechanisms [6, 7, 14, 20–23] (with the exception of the hornet *Vespa mandarinia*, which uses a pheromone signal to recruit nestmates to assist in attacking honey bee colonies [24]).

Foraging and recruitment to food have been little studied in the over 230 species of Neotropical swarm-founding wasps in the polistine tribe Epiponini [14]. This group is known for its broad range of colony sizes, complex social structure,

and striking ecological dominance [25, 26]. Several studies of *Polybia occidentalis* provide insight into the foraging process in this group. *P. occidentalis* is characterized by moderately large colony size and by the ability to store nectar in the nest [26, 27]. During the founding stage, foraging rates are directly correlated with the number of cells in the nest and with the number of larvae in later developmental stages, both of which are indications of colony demand [28].

The coordination of colony-level foraging in *P. occidentalis* occurs without the use of food-source scent-marking [29]. Instead, naïve foragers use cues encountered both in the nest and at the food site to help locate food. Direct introduction of a scented sugar solution into the nest causes an increase in the number of foragers departing from the nest [30]. At least some of these site-naïve individuals learn the food-associated scent and use it as a cue to help locate the source in the field [7]. In a study showing the importance of local enhancement as a cue, foragers bringing a rich, unscented sugar solution to the nest caused an increase in the number of new individuals arriving at a feeding station, where they overwhelmingly chose the food dish at which conspecifics were feeding over an identical dish without conspecifics [6].

In two later studies on this species [7, 30], done at the same field site as the present study, large amounts of scented sugar solution were added directly to the nest, rather than letting foragers bring the resource to the nest from a dish to which they were trained. This approach has two advantages: (1) the large influxes of food stimulated a foraging response that rose enough above background rates to be quantifiable and (2) the output response could be precisely quantified in terms of the known amount of resource input. These studies also demonstrated that the recruiting effects occur independently of the behavior of returning foragers in the nest.

However, these prior studies leave several questions unanswered. Schueller et al. [7] added 40 mL of a 2.0 M sucrose solution to the nest, an amount equivalent to more than 6,000 crop loads (average forager crop size is approximately 6.6 μL) [31]. This translates to a foraging rate equivalent to over 100 loads/min during the 60 minutes of the study. As this rate is more than 35 times the maximum foraging rate documented for this species [32], it leaves open the question of whether the much smaller amounts of scented solution brought back by a few foragers can have the same inducing effect. Although Hrncir et al. [6] let trained foragers return to the nest from a distant dish, because they used unscented sugar solutions, no inferences could be made about the wasps' use of odor cues. Finally, none of these studies directly addressed the question of whether returning foragers provide information about the direction of the resource. In many swarm-founding wasp species, during the dispersal of a swarm from its parental nest, scout wasps lay scent spots on vegetation between the old nest and the new site they have chosen [26, 33, 34]. Although some *Agelaia* spp. have been shown not to use this system to recruit nestmates to a rich protein source [20], whether or not in *P. occidentalis* uses this type of recruitment system for either protein or carbohydrate resources has not been tested.

Here, we address three questions. Firstly, we ask whether the influx of a profitable, scented carbohydrate resource brought to the nest by active foragers is sufficient to induce inactive foragers to begin foraging and searching for a resource with the same scent. Secondly, we ask whether more foragers arrive at the resource being exploited by the active foragers than at an identical resource in a different direction, which could indicate that directional information is being conveyed at the nest. Finally, we ask whether the magnitude of the colony's response to the influx of high-quality food is correlated with colony demand, as measured by number of adults and/or brood, and how the colony-level response is parsed at the individual level.

2. Methods

The investigation was conducted on the private property of the Hagnauer family and the adjacent grounds of Hacienda La Pacifica, near Cañas, Guanacaste, Costa Rica (10.450N, 85.125W), during June and July 2010, shortly after the beginning of the rainy season. Historically, this area was dry forest. Now the Hagnauer property is predominantly pastureland with scattered trees, whereas the grounds of Hacienda La Pacifica have wooded patches with openings planted to lawns and ornamentals. *P. occidentalis* nests are abundant in such disturbed areas; the wasps often construct nests in trees and shrubs along fence lines or roadsides. Colonies were sought within a radius of approximately 1 km from the study site and then moved to locations convenient for conducting the experiment, typically into isolated trees in pastures or yards. We conducted thorough searches of potential nest sites in the area to be sure that no other *P. occidentalis* colonies were close enough for their foragers to interfere with our experiments. Because these wasps are unable to fly in the dark, nests were moved at night to ensure that all colony members were present in the nest during the relocation process. At their new locations, nests were attached to branches about a meter high using metal binder clips, wire, or plastic zip ties. The proximal ends of the branches were coated for approximately 2.5 cm with Tanglefoot (Tanglefoot Co., Grand Rapids, MI, USA) to prevent predation by ants.

Two feeding stations were used during this investigation. The training station was used to mark foragers and, after marking was completed, was used to supply a sucrose solution scented with the training scent to trained foragers. The testing station offered a choice of scented sucrose solutions, one with the training scent and another with an alternate scent. Both stations consisted of Plexiglas tabletops (43 cm $\times$ 30 cm) attached to tripods and adjusted so that the dishes were approximately 0.75 m above the ground and clear of nearby vegetation. The training and testing stations were located 5 m apart and 5 m downwind from the colony (Figure 1). A 2.0 M sucrose solution, either unscented (during training and marking) or containing a 2% scent extract (during testing), was used in all dishes. The sucrose solution was placed in glass feeder dishes (6.5 cm diameter, 1.75 cm deep)—one placed in the center of the training station, and two placed approximately 11 cm apart at the

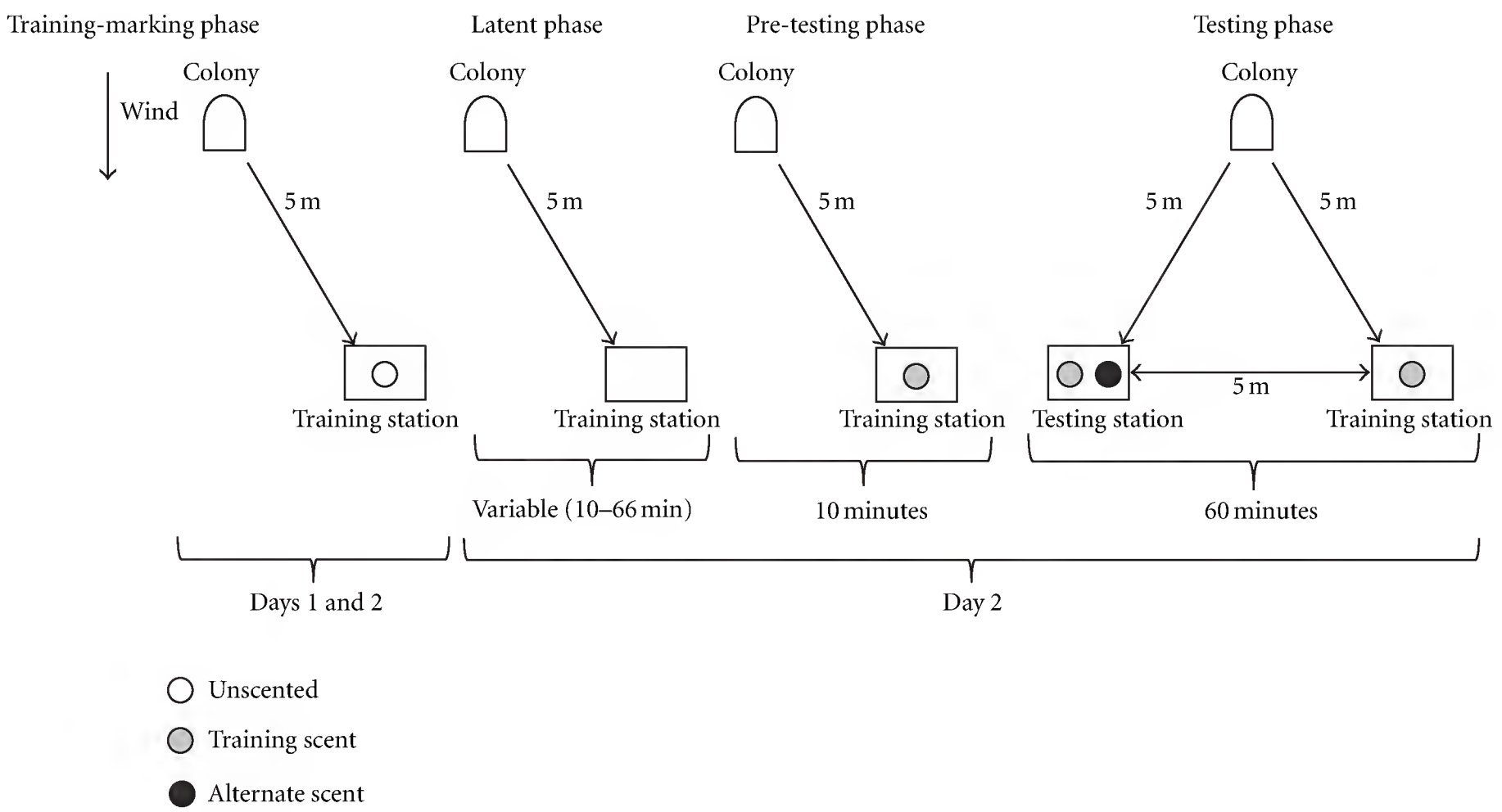

FIGURE 1: Experimental set-up and procedure. Prevailing wind direction is indicated.

testing station. For the scented solutions, extracts of pure vanilla and imitation cherry (McCormick & Co, Inc., Hunt Valley, MD, USA) were used. Throughout the investigation, the criterion of a "visit" to a dish required that the forager land on the dish and imbibe some of the sucrose solution.

Each of seven colonies was used for one experimental trial (Table 1), consisting of a two-day training-marking phase, a latent phase, a 10 min pretesting phase, and a 60 min testing phase (Figure 1). During the training-marking phase, a large pool of foragers were trained to forage at a dish containing an unscented sucrose solution at the training station and were marked so that they could be identified during the pretesting and testing phases (Table 1). The training procedure began by placing the dish against the nest, allowing workers to feed from it, and then incrementally moving it to the final experimental location (procedure described in detail in von Frisch [35] for honey bees and Schueller et al. [7] for *P. occidentalis*). By the time the training dish was moved to the final location, 5 m downwind of the nest, many foragers were foraging from it.

For marking, each forager was caught by grasping her around the petiole with reverse-action forceps as she landed on the training dish. She was then immediately marked on the thorax with a unique color code (with Decocolor paint pens) and released. The total numbers of individuals marked during both training-marking phase days are given in Table 1. On day one marking continued until, for a period of 10 min., all foragers arriving at the dish were already marked; in other words until there were no longer any unmarked foragers arriving. At the beginning of day two of the training-marking phase, additional unmarked foragers arrived at the dish. These, too, were marked until there were no more unmarked foragers arriving at the training station. The ultimate location of the training station was the same for all phases of the experiment. For each colony the training-marking sessions were carried out on consecutive days, except for colonies 10014 and 10004, when rain intervened (Table 1).

When marking on day two was complete, the training dish was removed from the table at the training station, defining the beginning of the latent phase. With a reward no longer present, the rate of forager arrivals at the station waned. The latent phase ended when no foragers arrived for a period of 2 min. The duration of the latent phase ranged from 10 to 66 min (Table 1). The purpose of the latent phase was to allow foraging on the unscented solution to cease before the colonies were exposed to the training scent.

At the end of the latent phase, the 10-min. pretesting phase was initiated by placing a feeder dish containing 2.0 M sucrose solution, now scented with the training scent, atop the training table. For each colony, one scent was randomly assigned as the training scent and the other as the alternate scent (Table 1). The purpose of this phase was to allow marked foragers to resume foraging, only now they were bringing the training-scented sucrose solution to the nest. Their identities and numbers of visits to the training dish were recorded. All unmarked foragers arriving at the training dish were counted and then captured by placing a 15-dram plastic collection vial over them when they landed on the dish or table. They were placed on ice until the end of the experiment, then released. This was done to ensure that the identity of each forager arriving at the training dish was known and that no forager foraged at both the training and testing dishes.

At the end of the pretesting phase, the 60-minute testing phase was initiated by placing two dishes (henceforth

Table 1: Colony information. For each colony the following are indicated: the colony identification number, dates on which foragers were marked and tested, training scent used, number of combs in the nest, number of adults present at collection (within 36 hrs of end of experiment), total number of foragers marked during the training-marking phase, and the duration of the latent phase. Colony 10028 absconded before it was collected, so it was not possible to count the number of adults on the nest.

Colony	Dates	Training scent	Number of combs	Number of adults	Total marked	Latent phase (min)
10052	June 25-26	cherry	7	2120	93	16
10048	June 28-29	vanilla	7	409	68	10
10029	July 2-3	cherry	7	710	33	25
10014	July 8, 10	vanilla	6	1322	62	24
10028	July 11-12	vanilla	12	NA	66	15
10018	July 15-16	cherry	7	572	36	66
10004	July 21, 23	vanilla	6	462	20	45
Total					378	

referred to as "testing dishes") containing a 2.0 M sucrose solution on the table at the testing station. The testing dishes were placed 11 cm apart and crosswind from each other. One dish was scented with the training scent and the other with the alternate scent. All three dishes—the dish at the training station and the two at the testing station—remained in place throughout the testing phase. At the training station, marked foragers were free to make repeated visits to the dishes; their identities and the number of trips each made were recorded. At the testing station, we recorded the identity, time of arrival, and dish choice (scent and position—left or right, as seen from downwind) of each forager. After each arrival by a forager at a testing dish, the position (left versus right) of the training-scented dish was randomly assigned using a random number table. To ensure that each naïve forager arriving at the testing station made only one visit and had not previously foraged at the training dish during the pretesting and testing phases, as well as to prevent the alternate scent from being brought to the nest, all foragers, marked and unmarked, arriving at either of the testing dishes were captured in 15-dram plastic collecting vials and placed on ice until the end of the experiment. Foragers were defined as "naïve" if they had not visited the training station while the scented dish was present (i.e., during the pretesting and testing phases), and so had no experience with the training scent other than potential exposure to it on the nest. By the end of the testing phase, ample sucrose solutions remained in all dishes.

Within 36 hrs after the conclusion of its testing phase, each colony was collected after sundown by enclosing it in a plastic bag and snipping the supporting branch. The bagged nest was placed in a freezer for at least 12 hours to kill the adult wasps, and then counts were made of the number of adults present, the number of combs in the nest, the number of brood cells, the number and approximate stages of the larvae, and the number of pupae. The adults from colony 10028 absconded before collection, so the adults could not be counted.

Analysis of forager choice at the testing station was performed using a one-factor ANOVA model. We used the proportions of landings at the two dishes at the testing station, rather than absolute counts of landings, because the colonies differed in size and therefore it was not meaningful to compare absolute counts. The proportion of landings on the dishes depended both on what scent was used and whether or not the scent was the one to which the wasps were trained. In order to isolate the effect of training, particularly with a different number of trials conducted with each scent as the training scent, our analysis had to account for both factors. To do so, we define Y_{ij} as the proportion of landings on the dish containing vanilla, whether or not it was the training scent. The model underlying our analysis can be written as

$$Y_{ij} = \mu + T_i + e_{ij}, \tag{1}$$

where $i = 1, 2$ corresponds to the training scent (1 = vanilla, 2 = cherry); j indexes the trial number for each level of i; μ is the overall mean; T_i is the treatment effect; e_{ij} is the random error. We used a weighted ANOVA (with weights proportional to total number of arrivals at the testing station), recognizing that a trial with more landings will result in a more precise measure of the proportion. Because the response variable is a proportion, we used the "arcsine square-root" transformation to achieve homogeneous variance [36].

We predicted that if marked foragers are communicating directional information back at the nest, significantly more naïve foragers would arrive at the training station. A paired t-test was used to compare the number of naïve foragers arriving at the training station to the number arriving at the testing station.

Our analysis of foraging-rate patterns during the pretesting and testing phases explored the relationships between various pairs of the variables: total forager arrivals, average number of trips per forager, number of marked foragers at the training stations, total number of adults, number of larvae, and latent phase. Where appropriate, simple linear regression analyses were performed to quantify the relationships.

Five additional experimental trials were performed, but results were excluded from the forager-choice analysis and/or foraging-rate analyses. For three of these colonies, the total number of arrivals at the testing station was one or none, and thus the colonies provided insufficient data for either analysis. During another trial, the marks on the majority of

Table 2: Arrivals at the training and testing stations during the pretesting and testing phases. Column 2: number of marked foragers making one or more trips to the training station. Columns 3–11: numbers of arrivals. Only the marked foragers visiting the training station (column 3) were allowed to make repeated visits. All others were captured and held on ice upon arrival; therefore, columns 4 and 6–11 represent both numbers of arrivals and numbers of individuals arriving.

		Training station			Testing station					
		Number of arrivals			Number of arrivals					
		Training scent			Training scent			Alternative scent		
(1) Col	(2) Number mk'd indiv	(3) Mk'd	(4) Unmk'd	(5) Total	(6) Mk'd	(7) Unmk'd	(8) Ttl	(9) Mk'd	(10) Unmk'd	(11) Ttl
04	6	197	16	213	3	0	3	1	0	1
18	7	360	8	368	1	6	7	1	1	2
29	8	270	3	273	0	3	3	0	0	0
14	11	204	5	209	1	2	3	1	1	2
28	14	266	16	282	3	3	6	0	3	3
52	22	185	1	186	3	6	9	2	4	6
48	33	179	10	189	1	3	4	0	3	3
Ttl	101	1661	59	1720	12	23	35	5	12	17

Table 3: The relationship between the total number of arrivals to the training station and the average number of foraging trips/forager to the training station, the number of adults in colony, the number of marked foragers foraging at the training station, the number of larvae in the colony, and the duration of the latent phase, using a simple regression analysis.

Source	Slope	Adjusted R^2	*df*	*P*
Average number of trips/forager	3.71	0.84	6	0.0009
Number of adults	0.030	−0.13	5	0.594
Number of marked foragers foraging at training station	−5.56	0.27	6	0.108
Number of larvae	0.044	−0.023	6	0.395
Latent phase (min)	2.22	0.34	5	0.095

foragers arriving at the testing station indicated that they were members of a nearby, newly relocated swarm and not from the colony being tested. The fifth trial (colony 10052) was excluded because its results appeared to have been biased by a previous trial using the same colony. This colony was tested on June 26 with cherry as the training scent and again on July 1 with vanilla as the training scent. Sixty-seven percent of the foragers coming to the testing dishes on July 1 chose the cherry-scented solution. Since *P. occidentalis* colonies store nectar [6, 27], it was likely that colony members had been exposed to the scent of cherry for five days and thus were apt to search for the cherry rather than the newly introduced vanilla. Although this trial was not used for the forager-choice or latent phase analysis, it was included in foraging-rate analysis because nectar storage is not expected to affect foraging rates. Colony 10028 was excluded from regression analyses involving total numbers of adults because the colony members absconded before it could be collected and censused.

3. Results

3.1. Training Station. One-hundred-one marked foragers from the seven colonies made a total of 1,661 trips to the training dish during the pretesting and testing phases (mean = 16.4, SD = 17.3, max/min = 65/1) (Figure 3; Table 2). In addition, fifty-nine unmarked foragers arrived at the training dish during the pretesting and testing phases (Table 2).

Table 4: Relationship between the total number of foraging trips to the training dish/forager and latent phase (min), the number of adults in the colony, and the number of larvae in the colony, using a simple regression analysis.

Source	Slope	Adjusted R^2	*df*	*P*
Latent phase (min)	0.74	0.80	5	0.0039
Number of adults	0.0050	−0.17	5	0.726
Number of larvae	0.012	−.029	6	0.404

The distributions of the numbers of foraging trips made by marked foragers from each nest to the training station for each colony during the pretesting and testing phases are shown in Figure 3. The total number of arrivals to the training station, a measure of colony foraging effort, is correlated with the average number of foraging trips/forager, but not with the number of marked foragers foraging at the training dish, the total number of adults in the colony, or the number of brood in the second larval instar and above (Figure 4, Table 3). The number of foraging trips to the training dish per forager is correlated with the duration of the latent phase, but not the number of adults in the colony, or the number of larvae in the colony (Figure 5, Table 4).

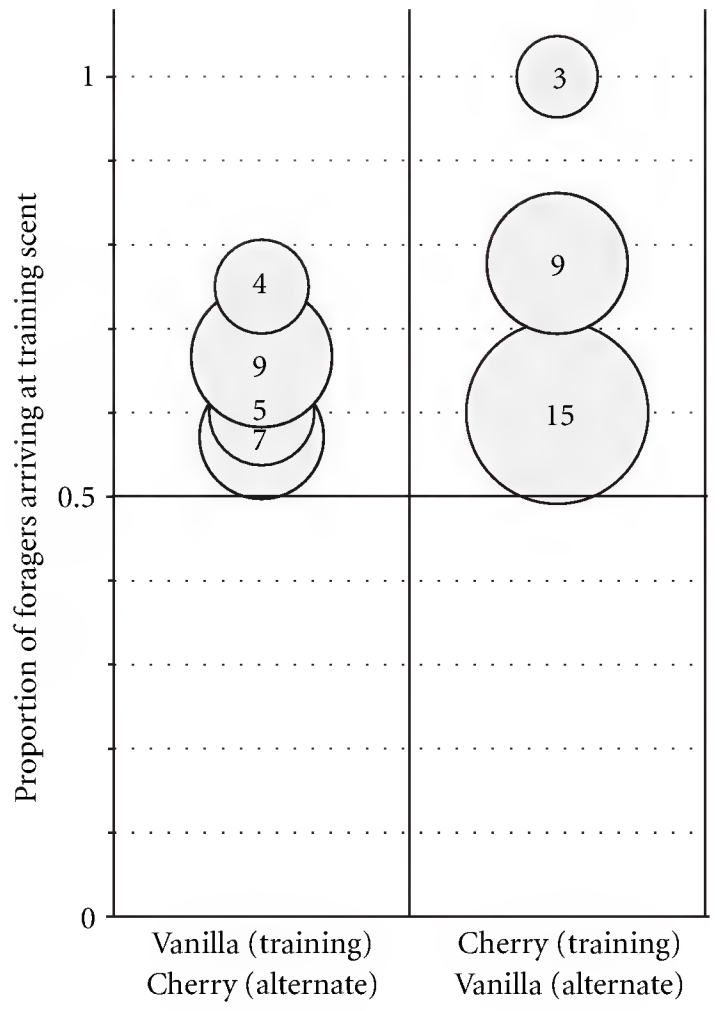

FIGURE 2: Arrivals at the testing station. Training and alternate scents are indicated. Each circle represents one colony. The center of the circle indicates the proportion arriving at the training scented dish for the colony. The area of the circle is proportional to the total numbers of unmarked foragers arriving at either of the dishes at the testing station; this number is indicated by the number inside the circle.

3.2. Testing Station. Fifty-two foragers from the seven colonies arrived at the one or the other of the two testing dishes during the testing phase (Table 2). Of those, thirty-five were unmarked, so had not previously foraged from any of the dishes (Table 2). Across all colonies, two-thirds of the foragers that arrived at the testing station chose the training scent over the alternate scent $F1, 5 = 7.32, N = 7, P = 0.0425$ (Figure 2).

The average time between forager arrivals at the testing station was 373 seconds, SD = 531, max/min = 2741/21 seconds, $N = 45$ (7 foragers were the first arrivals so could not be used for this analysis). None arrived while another was at the station.

The number of unmarked foragers arriving at the training station (59) did not differ significantly from the total number of naïve foragers arriving at the testing station (52) (paired t-test: $df = 6$, $T = -0.327$, $P = 0.755$), or the number of unmarked foragers arriving at the testing station (35) (paired t-test: $df = 6$, $T = -1.149$, $P = 0.294$). No marked forager foraged at both the training and testing stations during the testing phase. Only 118 (31%) of the 378 foragers that were marked during the training-marking phase arrived at the stations during the pretesting and testing phases (Tables 1 and 2).

4. Discussion

In this investigation, naïve foragers learned the scent of a carbohydrate solution brought to the nest by successful foragers and used the food-scent cue to locate a sucrose solution at a novel location off nest. Foragers brought the scented sucrose solution to the nest in amounts many times smaller than those added artificially to nests in a previous study [7] and represented a naturally attainable rate of food influx into the nest.

Newly-activated foragers arrived in equal numbers at the training and testing stations, suggesting that these foragers searched for a familiar resource off nest without obtaining directional information at the nest. This result also fails to provide any evidence that *P. occidentalis* lays scent-marks to rich food sources, as it does to new nest sites [26, 33, 34]. Because there was never a wasp present when a forager arrived at the testing station, foragers could not have been using local enhancement to make a choice between the dishes. We conclude, therefore, that naïve foragers relied upon olfactory cues to locate the scented solution off nest. This is the first demonstration that *P. occidentalis* foragers are activated to visit a novel feeder location in response to foragers bringing a scented resource to the nest, without having been trained to arrive at that specific location [6, 7] or using local enhancement cues [6].

The strength of volatiles characterizing natural carbohydrate sources utilized by *P. occidentalis* no doubt varies. Honeydew and extrafloral nectars may provide relatively weak olfactory cues, whereas floral nectars and ripe and rotting fruits no doubt provide strong cues that are detectable from some distance downwind [37]. It is likely that both olfactory cues emanating from a fruit and the visual cue of conspecifics already foraging on it are both utilized by searching foragers, but their relative importance may vary depending on a number of variables, including wind strength and direction and line-of-sight visual distance.

How effective were the active foragers in bringing naïve wasps to the resources? Across all colonies, the 1,661 trips to the training dishes by marked foragers resulted in 111 foragers arriving at the training and testing stations. Thus, it took 1,661/111 = 15.0 forager loads of 2.0 M sucrose to bring each new forager to the resources. By comparison, when Taylor et al. [30] applied 4 mL, or approximately 600 forager loads [30, 31], of the same resource (2.0 M sucrose) directly to the nest, it resulted in approximately 36 additional foragers exiting the nest. By the same calculation, it required 600/36 = 16.7 forager load equivalents to stimulate one extra departure. Taylor et al. [30] counted new foragers as they left the nest, whereas in our investigation the new foragers were counted as they arrived at the resources 5 m downwind from the nest. Since it is unlikely that all naïve foragers departing in search of the scented resource experienced in the nest succeed in finding the resource, the fact that the two figures are so similar suggests that foragers returning with a rich resource (this study) may provide some form of alerting signal that stimulates more to leave the nest than would in response to the resource alone [30]. Although this comparison of the

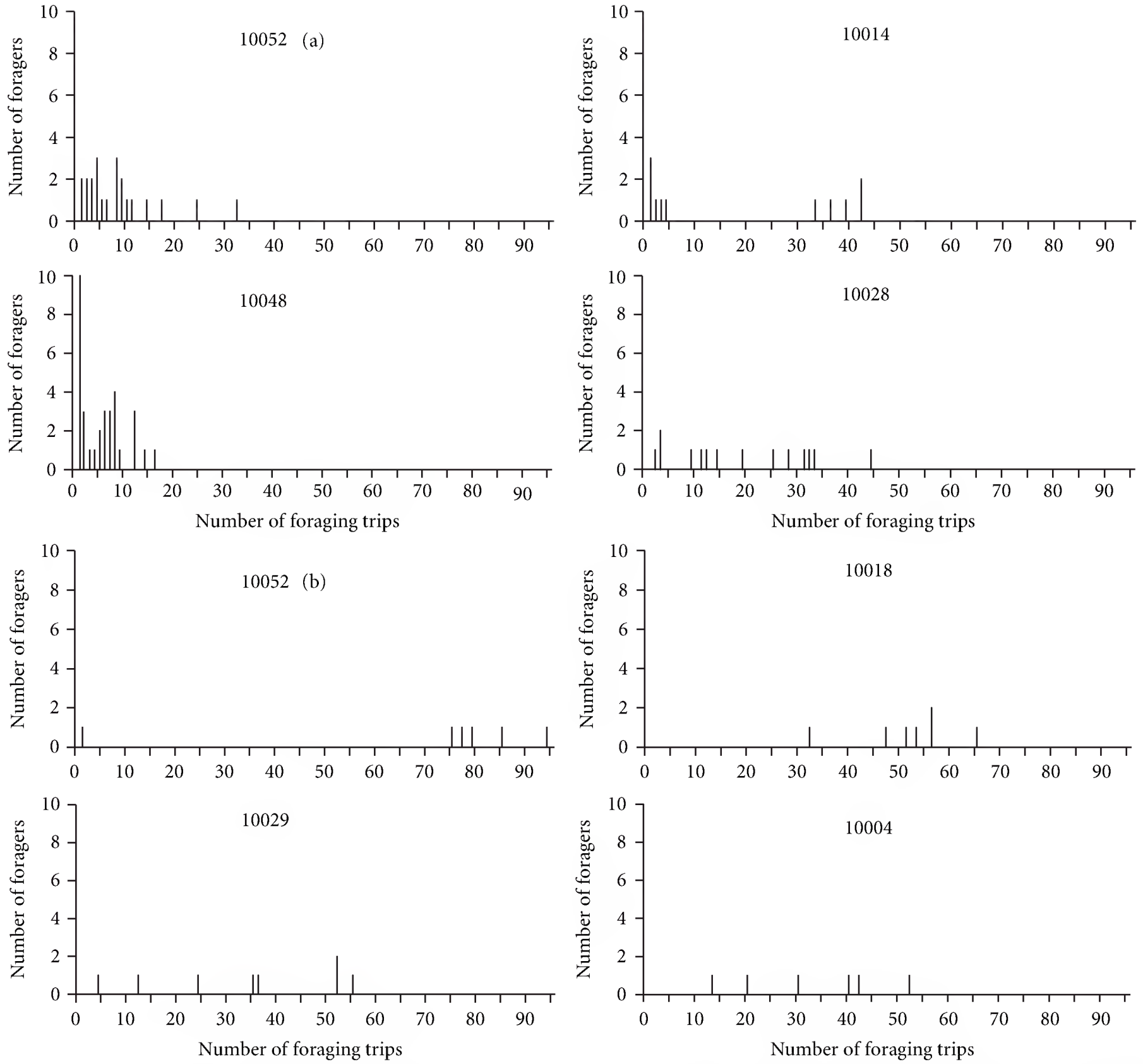

FIGURE 3: Distribution of numbers of foraging trips each marked forager made to the training station during both the pretesting and testing phases. The x-axis represents the number of trips by each forager and the y-axis represents the number of foragers making the indicated number of trips. 10052 (a) refers to colony 10052 when it was tested on June 26 and 10052 (b) refers to colony 10052 when tested again on July 1.

results of the two studies is rather crude, it is nonetheless worth making for the experiments it may suggest.

Foraging effort, measured as the total number of foraging trips made by marked foragers, did not correlate with colony demand, as measured by either the number of adults or the number of larvae in the colony. This result contrasts with that of Howard and Jeanne [28], who found a strong correlation between foraging rate and colony demand in the same species. The difference may be attributable to differences in the methods used. Howard and Jeanne measured ongoing foraging rates in unmanipulated colonies [28], whereas we trained a set of foragers to make repeated visits to a rich resource. Foraging rates vary tremendously among individuals [38] for reasons that are not well understood. The overall rate at which each colony exploited the resource may have been a function of the individual rates of those foragers in the subset that happened to have been trained to our dishes, independently of the demands of the colony.

Given that colony-level foraging effort bore no relation to colony demand, nor to the number of active foragers in the colony, measured as the number of marked individuals arriving at the testing station, one might then expect a positive correlation between the number of foragers making repeat visits in each colony and the total number of visits to the dishes made by each colony. In contrast to expectation, the relationship trended negative, although not quite significantly so. Although the regression of total trips per colony on the number of active foragers was not significant, there was a positive correlation between total trips and the average number of foraging trips/forager for each colony.

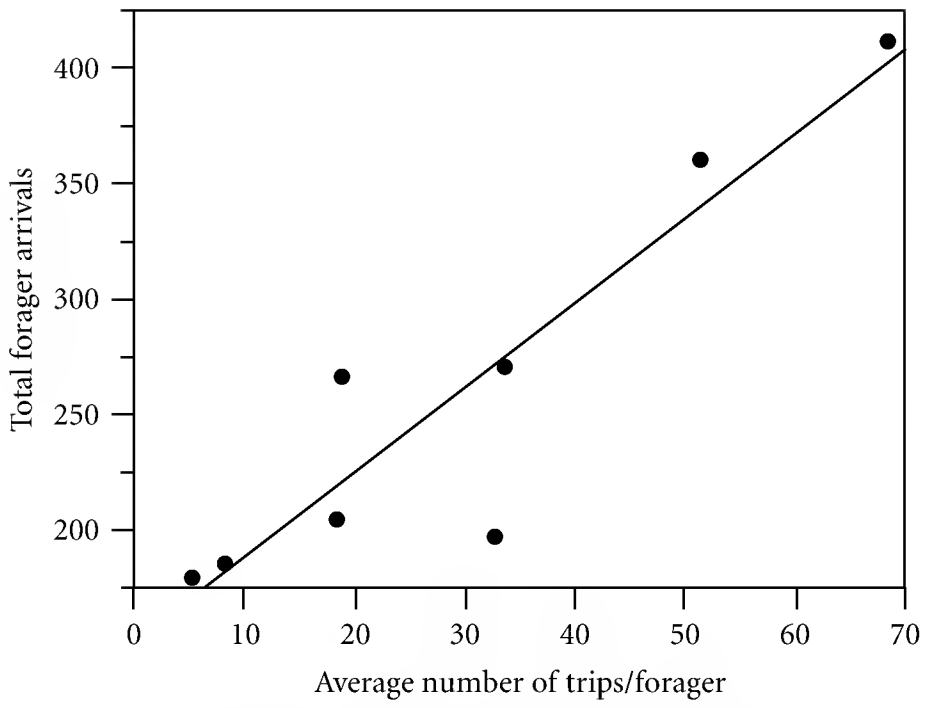

FIGURE 4: The total number of arrivals of marked foragers, a measure of colony-wide foraging effort, at the training station during the pretesting and testing phases as a function of the average number of trips per forager.

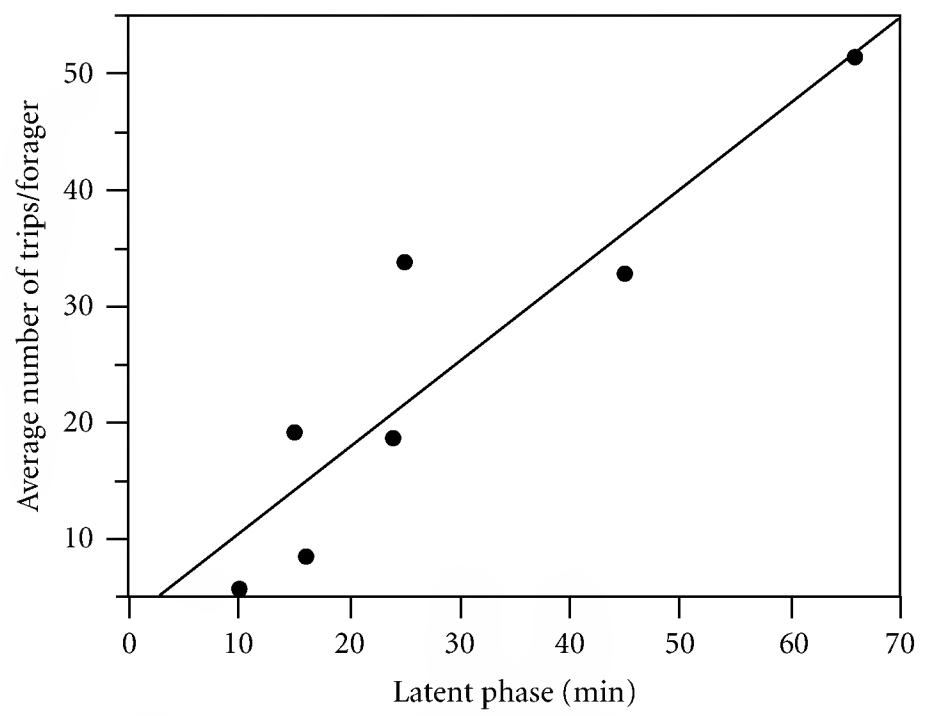

FIGURE 5: Average number of trips/forager for marked foragers arriving at the training station during the pretesting and testing phases as a function of the duration of the latent phase.

That is, in the colonies that employed the largest number of foragers, those foragers performed the fewest foraging trips, on average. In fact, this relationship was so strong that in colonies employing the fewest foragers, not only did each forager make more trips than in large-group colonies, but the total number of trips made by these colonies was almost double those in the large-group colonies. This is a counterintuitive pattern. A reasonable a priori assumption would be that samples of 6–33 foragers (Table 2) would have similar mean per-capita foraging rates, and therefore that the overall number of trips made by those samples would be in direct relation to the number of foragers in the sample. That this was not the case suggests the existence of some kind of group-size-related dynamic.

One possibility for the observed pattern is that, compared to colonies with small numbers of foragers, in colonies with a large number of foragers returning to the nest, each forager received a less-than-enthusiastic reception by nectar receivers, or a longer unloading-delay, causing them to wait longer before making the next trip. Another possibility is that colonies with the highest overall foraging rates had a higher proportion of "elites" actively foraging than the colonies with lower overall rates. Elite foragers make a disproportionate number of trips [39]. Although the presence of elite nectar foragers has not been documented in *P. occidentalis*, they have been shown to exist in *Vespula germanica* [39], and it has been shown that some *P. occidentalis* make disproportionately more of the foraging trips for nest building materials [40].

Interestingly, the number of foraging trips per forager was also positively correlated with the length of the latent phase, which may be a reflection of the average persistence and foraging rate of the colony's foragers. During the latent phase, the training dish was removed from the training station, disrupting any foraging reinforcement until foragers were no longer arriving at the station. Colonies with the most active and persistent foragers would, therefore, have the longest latent phase. This pattern might also be explained by different proportions of actively foraging elites across colonies. If elite foragers, in general, not only make relatively more foraging trips than nonelites, but also tend to persist longer at the task [40], then one would predict that the latent phase for colonies employing more elites would be longer than those with fewer active elites, the pattern observed during this investigation.

Results of the present investigation demonstrate that the social wasp *P. occidentalis* uses a cue-based form of food recruitment whereby the arrival in the nest of foragers with a rich, scented carbohydrate resource induces naïve nestmates to forage at an off nest resource with the same scent. Many groups of social insects employ this simple mechanism [41]. In choice experiments, naïve *Vespula vulgaris* and *V. germanica* foragers chose resources with the same scent as the ones that were brought to the nest by successful foragers [22, 42]. When a scented carbohydrate solution was added directly to the nest, naïve foragers also preferentially chose the resources with the same scent off nest [23]. Similarly, bumble bee (*Bombus terrestris*) foragers preferentially chose resources with the same scent as those stored in honey pots inside the colony [17, 41]. Honey bee foragers use scent cues experienced at the nest, in addition to the waggle dance, to help locate food sources off nest [4, 15, 35, 43–45]. Cue-based recruitment to food sources has the advantage of allowing foragers to home in on particularly profitable resources, thus, expending less energy than independent searching would require [5, 45]. Recruitment is especially advantageous for species such as *P. occidentalis* that live in the tropics, where resources are more patchily and ephemerally distributed than in temperate regions [45, 46].

In light of the results from this and several other recent investigations, a good picture of carbohydrate foraging and recruitment for *P. occidentalis* can now be drawn. Foragers learn to associate visual and olfactory cues with a carbohydrate resource and use these to aid them in relocating the resource on return visits [7, 47]. They do not mark

the resource with a pheromone signal [31]. Meanwhile, the influx of carbohydrates from successful foragers incites inactive foragers to leave the nest and search off nest. They obtain no directional information from the successful foragers, but they do learn the olfactory cues of the food brought to the nest and search for a resource with the same scent ([6, 30], present investigation). Local enhancement is a second site-based cue that attracts nestmates visually to a specific resource [6]. Both kinds of cues are likely to shorten search time, thereby decreasing energetic expenditures and mortality, by allowing foragers to home in on high quality resources more quickly than by uninformed random searching ([7, 30, 48, 49], present investigation). This mechanism of activating previously inactive foragers may aid in rapid exploitation of resources when they become available [30].

One of the lingering questions about foraging in *P. occidentalis* is whether or not signals play a role in the recruitment process. Taylor et al. [30] observed an increase in rapid running across the envelope when a carbohydrate resource was added to the nest. This behavior was described as being much like the wing fanning and animated running signals used by successful *Bombus* spp. foragers that induce nestmates to begin foraging [50–53] and may indicate that *P. occidentalis* uses an alerting signal as well as a cue-mediated recruitment mechanism [30]. This has yet to be confirmed [30] but, if used, may serve to enhance the efficacy of cue-based recruitment by activating more individuals than is possible via cues alone.

Acknowledgments

This project would not have been possible without the generosity of the Hagnauer family and the staff at Hacienda La Pacifica in allowing the authors to conduct research on their properties. The authors give sincere thanks to Evan Brus for assistance in the field. Erik Nordheim offered valuable guidance with statistics, and Benjamin Taylor provided helpful comments and suggestions on the paper. Research was supported by the Zoology Department, University of Wisconsin-Madison. T. I. Schueller was supported by a Dickie Family Sauk County Educational Award. Neither T. I. Schueller or R. L. Jeanne has a financial relationship with Decocolor, Tanglefoot, Plexiglas, or McCormick & Co. or any other commercial entity referenced in this paper.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 382938, 5 pages
doi:10.1155/2012/382938

Research Article

A New Species of Neotropical Carpenter Ant in the Genus *Camponotus* (Hymenoptera: Formicidae), Apparently without Major Workers

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Received 19 April 2012; Revised 4 June 2012; Accepted 11 June 2012

Academic Editor: Martin H. Villet

We describe a new species of carpenter ants from Ecuador, which apparently has an obligatory relationship with the ant plants *Cecropia membranacea* Trécul, *C. herthae* Diels and *C. marginalis* Cuatrec. The workers are relatively small and hairy, and based on a number of collections, it does not appear to have major workers. We compare the new species to *Camponotus balzani*, to which it appears to be similar and which has normal major workers, and also lives in *Cecropia* spp.

1. Introduction

Camponotus carpenter ants have a worldwide distribution and form one of the hyperdiverse genera together with *Pheidole* [1]. It is still unknown which of these two largest genera has the greater number of species as many new species await discovery. In the New World, about 650 taxa have been named in *Camponotus*, which will be reduced to about 440 valid species, including about 140 new species (Mackay, unpublished, manuscript available at http://www.utep.edu/leb/antgenera.htm). Other species of neotropical *Camponotus* have been recently described [2–4].

The revision of such a large group has taken nearly 20 years, with several more needed to complete the work. Occasionally names are needed for ecological studies, which cannot wait the completion of the entire work. We here describe an interesting new species to provide a name for an ant whose interaction with *Cecropia* will be described by the junior author in future papers.

This species is unusual in that the workers appear to be monomorphic. The majority of the carpenter ants are dimorphic, but monomorphic workers are also found in the *montivagus* species complex [5, 6], subgenus *Dendromyrmex* [7], and some of the species in the subgenus *Myrmobrachys* are only weakly dimorphic.

Placement of this species into a subgenus is difficult because of the lack of major workers. Based on its similarity to *C. balzani* and several characters listed below, we placed it in the subgenus *Tanaemyrmex*. The clypeal carinae of the minor worker, female, and to some extent the male are well developed. The sides of the clypeus diverge anteriorly in all three castes. The head of the minor worker is longer than broad, with large eyes that reach the sides of the head. The minor worker is relatively large, with an elongated mesosoma, which is typical for the subgenus *Tanaemyrmex*. The dorsum of the mesosoma of the minor is convex and rounded and somewhat angulate between the two faces. The propodeal spiracle is elongated. The petiole is somewhat thickened (viewed from side). Appressed pubescence is sparse. The head and mesosoma are coriaceous, and the gaster is finely striolate. These characteristics are similar to most other species considered to be in *Tanaemyrmex*.

2. Materials and Methods (Measurements and Indices)

At least ten individuals of each of eight species of *Cecropia* were found that were at least 100 m apart. The exceptions were *C. litoralis* Snethl, in which only two individuals were found and *C. marginalis* where eight plant individuals were collected. *Camponotus reburrus* was found in saplings of *Cecropia membranacea* and *C. marginalis*, but the majority of samples came from *C. herthae*. Seventy-five *Cecropia* individuals were collected, and *C. reburrus* was found in 9.3% of the trees collected.

Specimens were measured using an ocular micrometer in a dissecting microscope. The following abbreviations are used (all measurements in mm):

HL: head length, anterior of median lobe of clypeus to mid-point of posterior margin;

HW: head width, maximum excluding eyes;

SL: scape length, excluding basal condyle;

EL: eye length, maximum dimension;

CL: clypeal length, from tops of posterior lobes of clypeus to anterior median edge;

CW: clypeal width, measured at level of tentorial pits;

WL: weber's length, anterior border of pronotum to posterior border of lobe of metapleural gland;

FFL: front femur length (maximum);

FFW: front femur width (maximum);

CI: cephalic index, HW/HL × 100;

SI: scape index, SL/HL × 100 (note HL used instead of HW);

CLI: clypeal index, CLI, CW/CL × 100;

FFI: front femur index, FFW/FFL × 100.

3. Results

The new species is characterized as follows.

Camponotus (*Tanaemyrmex*) *reburrus* Mackay *n*. sp. (Figures 1, 2, 3, 4, 5, 6, 7, 8, and 9).

Diagnosis. The minor worker of *C. reburrus* is a relatively small (4.4–6.6 mm total length) yellowish brown specimen, with a transversely striped yellow and brown gaster, or at least with yellow lateral splotches. The sides of the head are straight and parallel, and the carina on the clypeus is well marked. The antennal scape has numerous erect hairs along the shaft, the hairs on the tibiae are coarse and suberect. Most surfaces are moderately to strongly shining.

The female of C. reburrus is a small (total length 6-7 mm) hairy specimen, which is shiny dark brown with lighter colored legs. The gaster has lateral yellow splotches. The sides of the head are straight and parallel. The size and abundant erect hairs on the head and scape should separate it from all others in the subgenus *Tanaemyrmex*.

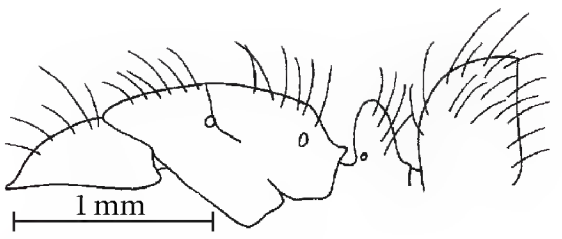

FIGURE 1: Mesosoma, petiole, and first gastral tergum of a minor worker of *C. reburrus* (Napo, Ecuador).

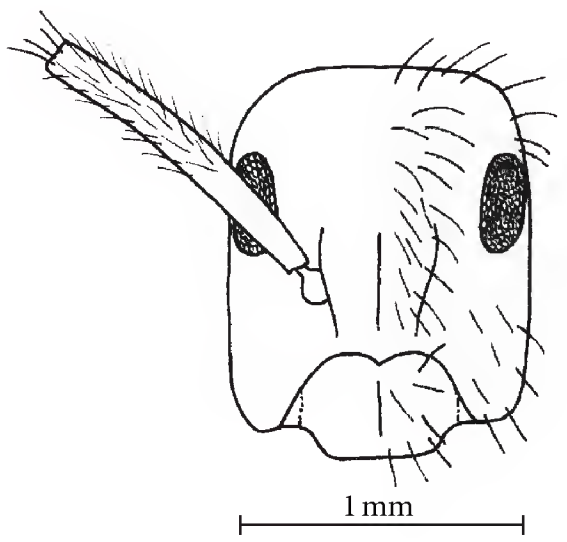

FIGURE 2: Head of a minor worker of *C. reburrus* (Napo, Ecuador).

The male of C. reburrus is a small (total length 4.0–5.5 mm) dark brown to yellowish brown specimen, with abundant hairs on most surfaces. Other than color and hairiness, it does not seem to possess characteristics which would distinguish it from other small males of *Camponotus*.

The major worker apparently does not exist.

3.1. Description of the Minor Worker. Minor worker measurements (mm, $n = 4$)*:* HL 1.20–1.38, HW 0.90–1.00, SL 1.06–1.14, EL 0.31–0.34, CL 0.36–0.45, CW 0.49–0.53, WL 1.64–1.84, FFL 0.96–1.08, FFW 0.30-0.31. Indices: CI 72–75, SI 83–88, CLI 108–145, FFI 29–31.

Mandibles with 6 teeth; anterior border of clypeus nearly straight, carina well marked; sides of head straight, parallel, posterior border convex; eyes extend slightly past sides of head; scape extends nearly 1/2 length past posterior lateral corner of head; mesosoma arched throughout, weakly depressed at sutures; dorsopropodeum about twice length of posteropropodeum, face weakly concave, spiracle circular; petiole moderately thickened when viewed in profile, apex rounded when viewed from front.

Erect hairs present on head, including surface of clypeus, cheeks, entire length of sides of head, posterior border, between frontal carinae, frons, ventral surface of head, scape with short (0.05 mm) erect and suberect hairs, erect hairs abundant on mesosoma, petiole and gaster, hairs on tibiae abundant and suberect; appressed pubescence sparse, tiny (up to 0.05 mm) hairs.

Head and mesosoma coriaceous, gaster transversely striolate, all surfaces moderately to strongly shining.

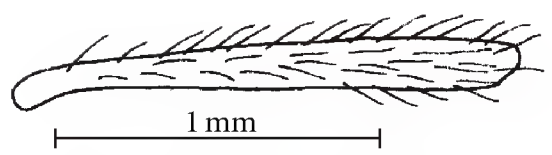

FIGURE 3: Posterior left tibia of a minor worker of *C. reburrus* (Napo, Ecuador).

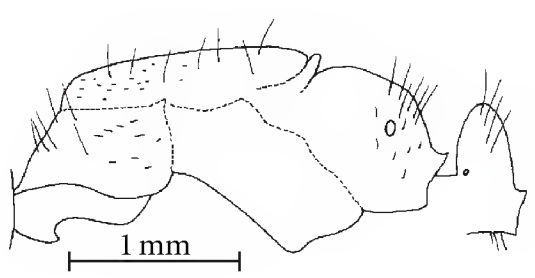

FIGURE 4: Mesosoma and petiole of the holotype female of *C. reburrus*.

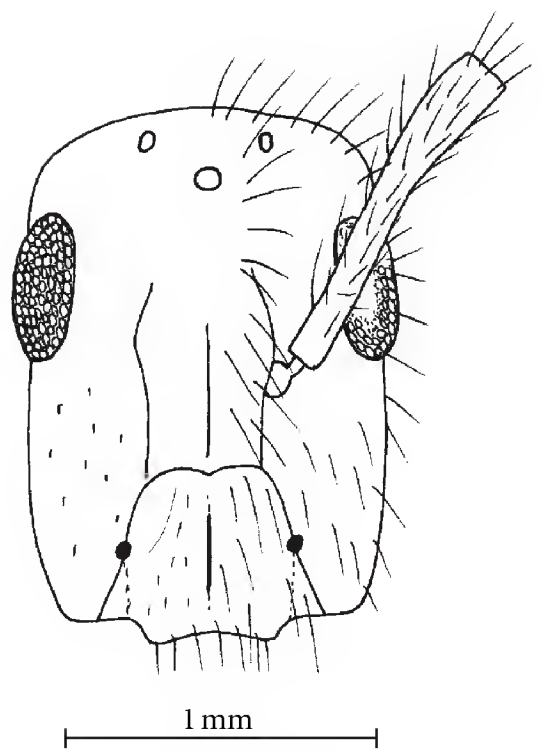

FIGURE 5: Head of the holotype female of *C. reburrus*.

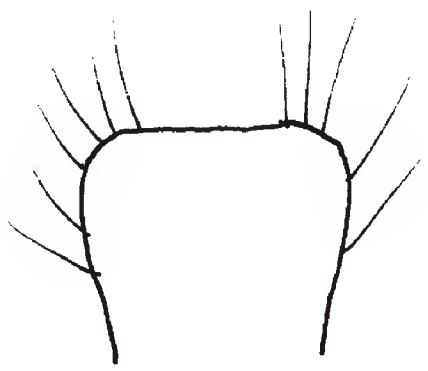

FIGURE 6: Petiole of the holotype female of *C. reburrus*, as seen from front.

Ferrugineous red, gaster with yellow lateral splotches ranging to striped brown and yellow.

3.2. Description of the Female. Female measurements (mm, $n = 4$)*:* HL 1.66–1.74, HW 1.22–1.26, SL 1.32–1.40, EL 0.51–0.54, CL 0.56–0.58, CW 0.58, WL 2.62–2.68, FFL 1.44, FFW 0.38-0.39. Indices: CI 72–74, SI 79–81, CLI 100–102, FFI 26-27.

Clypeus with carina poorly developed; anterior clypeal border concave, medial portion meeting lateral portion in rounded angle; frontal carinae closely placed; eyes large, extending past sides of head; scape short, extending past posterior lateral corner of head by 1.5 funicular segments; head elongate, rectangular with nearly parallel sides; posterior margin convex, rounded; mesosoma elongate; pronotum lengthened and lowered in profile; metanotal suture deep, propodeum lower than remainder of mesosoma, rounded; petiole thick in profile.

Erect hairs abundant, about 20 scattered over surface of clypeus, 5-6 on cheek, 5 or more on malar area, erect hairs on posterior margin and posterior lateral corner of head, scape with two distinct types of hairs, erect (or nearly erect) longer (0.13 mm) hairs, and not very abundant (fewer than 10, excluding similar hairs at apex), and suberect short (>0.08 mm) abundant (about 100) hairs, pronotum with long (0.4 mm) erect hairs along posterior border, erect hairs scattered on dorsum of remainder of mesosoma, petiole and gaster, femora with several nearly erect hairs, tibiae with numerous hairs that are mostly suberect; appressed pubescence sparse, short on head (0.02 mm), longer on gaster (0.06 mm).

Sculpture finely coriaceous, but strongly shining.

Color predominantly dark brown, legs lighter brown, coxae, trochanters and base of tibiae yellow, gaster with lateral yellow blotches on each tergum.

3.3. Description of the Male. Male measurements (mm, $n = 4$): HL 0.98–1.04, HW 0.84–0.86, SL 0.92–0.96, EL 0.39-0.40, CL 0.29-0.30, CW 0.34–0.36, WL 2.06–2.12, FFL 1.32–1.36, FFW 0.24-0.25. Indices: CI 83–86, SI 92–94, CLI 117–121, FFI 18.

Mandible without teeth, except apical angle; anterior border of clypeus broadly convex, clypeal carina poorly defined, but present; sides of head anterior to eyes concave, nearly parallel, posterior border of head broadly rounded, concave between ocelli; scape extending approximately 1/2 length past posterior lateral corner of head; eye large, occupying approximately 1/2 length of side of head; ocelli well developed; propodeal spiracle oval shaped; petiole thick when viewed in profile, apex slightly flat but convex as seen from front.

Erect and suberect hairs present on mandibles, clypeus, dorsum and ventral surfaces of head; sides of head, posterior border of head, dorsum of mesosoma, legs, petiole, and all surfaces of gaster; appressed hairs sparse few present on head, mesosoma, and gaster.

Dorsum of head coriaceous, mesosoma coriaceous but somewhat glossy, especially scutellum, side of pronotum, mesopleuron, gaster transversely striolate, moderately shining.

Pale medium brown, mandibles, cheeks, antennae, parts of scutum, legs yellowish brown.

Distribution. Known only from northeastern Ecuador.

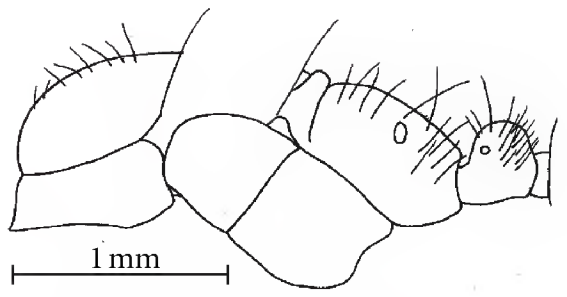

FIGURE 7: Mesosoma and petiole of a paratype male of *C. reburrus*.

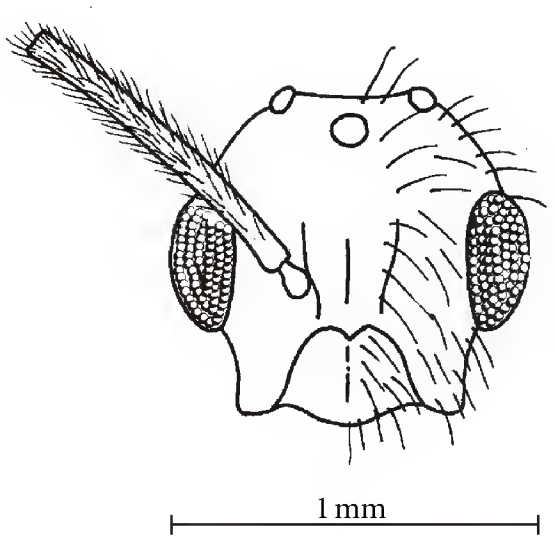

FIGURE 8: Head of a paratype male of *C. reburrus*.

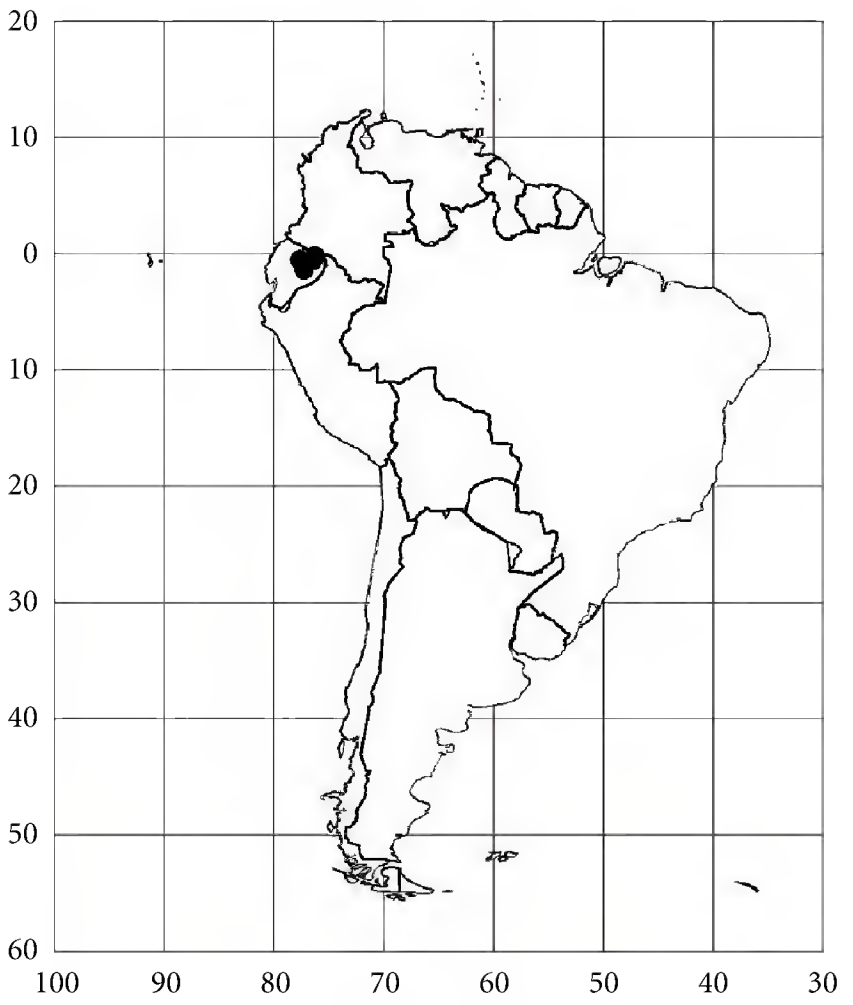

FIGURE 9: Camponotus reburrus.

Holotype Female. Ecuador, Napo, Sucumbíos, Sacha Lodge, 0.5°S 76.5°W, 290 m, 22-ii-1994, P. Hibbs (Los Angeles County Museum of Natural History); *Paratype females* (3) same data as holotype, 4-iii-1994, 20,30-ix-1994, 10,21-xi-1994 (Los Angeles County Museum of Natural History, Museo de Zoología, Pontificia Universidad Católica del Ecuador); additional *paratypes* including Orellana, Yasuni Research Station, 0°40′S 76°23′W, 200 m, 17-i-2009, C. Sagers and J. Trager numbers 2009-502, 2009-506, 2009-510, 2009-515 (26 workers, 1 dealate female, 4 alate females, 4 males), same locality, 19-i-2009, P. Barriga and G. Alvia numbers 2009-021, 2009-028, 2009-030, 2009-031, 2009-037 (58 workers, 3 females, 18 males). The female was selected as the holotype as the minor workers, and males of *Camponotus* spp. are often indistinguishable. Paratypes will be distributed to major South American, North American, and European museums, including the California Academy of Sciences, Collection of William and Emma Mackay, University of Texas, El Paso, Ernst-Moritz-Arndt-Universitat, Greifswald, Germany, Los Angeles County Museum of Natural History, Museum of Comparative Zoology, Harvard University, Museo de Historia Natural, Universidad Nacional de Colombia, Museo de Zoología, Pontificia Universidad Católica del Ecuador, Museu de Zoología da Universidade de São Paulo, Brasil, Muséum d'Histore naturelle, Genéve, Switzerland and the Naturhistorisches Museum Wien, Burgring, Austria.

Material Examined. Type series and **ECUADOR**: Napo, 24 km NE Archidona, 0°43′S 77°41′W, 1000 m, 7-viii-1991, P. Ward numbers 11394, 11396-2 (1 worker, 1 alate female, 1 dealate female, Museum of Comparative Zoology, Harvard University), 7 km ESE Puerto Misahualli, 1°4′S 77°37′W, 400 m, 5-viii-1991, P. Ward numbers 11366 (3 workers John Longino collection, 3 workers Museum of Comparative Zoology, Harvard University).

Etymology. From Latin, *reburrus*, meaning one with bristling hair, referring to the hairs on the head.

Comments. Camponotus reburrus does not appear to be closely related to any of the other described species in the subgenus *Tanaemyrmex*, other than *C. balzani*. This close relationship with *C. balzani* was further supported with DNA barcode analyses (Barriga, unpublished information).The minor workers can be separated in that the side of the head has few erect or suberect hairs (abundant in minors of *C. balzani*), the gaster is transversely striped with yellow bands (generally concolorous light brown in *C. balzani*, sometimes with a hint of tan transverse stripes), and the workers are slightly smaller (much overlap in size ranges in the two species). The females of *C. reburrus* are much smaller (maximum total length approaches 7 mm) than those of *C. balzani* (total length over 1 cm). The splotches or stripes on the gaster are definitely yellow in females of *C. reburrus* but are only pale brown (if present) on the gaster of females of *C. balzani*. The males of *C. reburrus* are also much smaller (total length approaching 4.7 mm) as compared to the males of *C. balzani* (total length greater than 5 mm). The males of *C. reburrus* are generally darker brown than the pale medium brown males of *C. balzani*. If major workers are found to exist, they will probably be similar to those of *C. balzani* with the sides of the head narrowed anteriorly, with a well-developed clypeal carina, but differing in having few erect hairs on the sides of the head. The gaster would be expected to have well-developed yellow splotches or bands, which

would differ from the major of *C. balzani* in which the bands on the gaster are only pale brown, if present.

The abundant erect and suberect hairs on the scape and the abundant erect hairs on the clypeus suggest affiliation with species such as *C. maculatus plombyi*, but minor workers of *C. reburrus* differ in being much smaller and in that the head is little narrowed anteriorly as in *C. plombyi*. *Camponotus reburrus* could be confused with *C. maculatus soulouquei*, but it has little in common with this latter species. *Camponotus reburrus* can easily be separated by the shape of the head of the minor, which has parallel sides in *C. reburrus* and as the sides of the head of the minor is strongly narrowed anteriorly in *C. maculatus soulouquei*.

The yellow blotches on the gaster may result in *C. reburrus* being confused with members of the subgenus *Myrmosphincta*. *Camponotus reburrus* can be easily separated as the surface is nearly smooth (rough and punctate in *Myrmosphincta*) and the head is rectangular shaped (narrowed anteriorly in *Myrmosphincta*). Thus, *C. reburrus* would not be easily confused with any other South American species.

Biology. Three of the winged female-type specimens of *C. reburrus* were collected in a malaise trap. Others were collected in *Cecropia* at the edge of secondary growth rainforest or in gaps inside primary growth rainforest. Among the *Cecropia* species where this species was found are: *C. marginalis* Cuatrec, *C. herthae* Diels, and *C. membranacea* Trécul. The ants were found inside *Cecropia* internodes and were not aggressive as are *Azteca* ants. Müllerian bodies and scale insects were found in the internodes where the ant species were living. This species is smaller than *C. balzani*, but the behavior inside *Cecropia* nodes is similar: the ants of both species do not attack but run and escape. *Camponotus balzani* and *C. reburrus* inhabit *Cecropia* juvenile and reproductive plants (plants with pistillate or staminate flowers), but most *Cecropia* species were found more commonly with aggressive colonies of *Azteca* ants.

Acknowledgments

The authors thank the Ernst Mayr fund of the Museum of Comparative Zoology of Harvard University for funding several trips to compare type material and to see the extensive collection of *C. balzani*. Gary Alpert provided lodging, and Stefan Cover allowed access to the ant collection. Gary Alpert will also put colored photographs of paratypes on the ant species website of the Museum of Comparative Zoology http://insects.oeb.harvard.edu/mcz/. The Ministerio del Ambiente of Ecuador, the World Wildlife Fund's Russell E. Train Education for Nature Program and the National Science Foundation, Office of International Science and Education Grant no. 0813594 provided funding for the project. They would like to thank an anonymous reviewer for correcting several mistakes and making important suggestions that improved the paper.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 925149, 6 pages
doi:10.1155/2012/925149

Research Article

Urban Infestation Patterns of Argentine Ants, *Linepithema humile*, in Los Angeles

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Received 21 February 2012; Revised 21 May 2012; Accepted 31 May 2012

Academic Editor: Brian Forschler

Infestations of buildings by Argentine ants, *Linepithema humile* (Mayr), were monitored on the campus of the University of California, Los Angeles. Foraging ant activity peaked during the hotter months of the year. The mean monthly maximum temperature, but not rainfall, positively correlated with indoor infestation frequency. Neither garden size nor the predominant groundcover vegetation correlated with the number of foraging ants at baits within gardens. Although the number of foraging ants outside a building varied over 40-fold, ant density in gardens did not predict the likelihood of infestation within the building. Also, the type of vegetative groundcover employed did not predict infestation frequency. There was, however, a significant negative relationship between the size of the garden outside of a building and the number of infestations. Given the large foraging area of *L. humile* workers, buildings next to small gardens may be infested simply because they lie within the "normal" foraging area of a colony. The best predictor of which rooms were infested within buildings was the presence of a water source. Thus providing water for ant colonies outside and away from buildings may be one method of integrated pest management to reduce the proclivity of ants to infest structures.

1. Introduction

Argentine ants (*Linepithema humile* (Mayr)) are a worldwide invasive exotic pest [1–3]. In urban areas that have a Mediterranean type climate they often invade dwellings and are one of the most consistently reported pest species [3–9]. Although *L. humile* tends to form high-density monocultures outdoors [10–14], it is not ubiquitous in its infestation patterns. Environmental factors such as mean yearly rainfall and minimum winter temperatures can broadly predict whether *L. humile* colonies can survive, but local abundances will be better predicted by idiosyncratic features such as human disturbance, native ant species presence, or irrigation regimes [15]. This variance suggests that there are identifiable, local factors that can also predict the likelihood of an infestation in buildings. One such factor could be features within rooms (e.g., food, water, and potential nest sites) that attract ants. A second variable could be the landscaping practices around buildings and their influence on ant activity and density [5, 9, 16].

The University of California, Los Angeles (UCLA) provides an urban setting in which infestations and their indoor and outdoor correlates can be measured. The ant fauna on the grounds is effectively limited to *L. humile* and buildings vary in how often ants invade them. Through the auspices of the university, access is available to the grounds and many of the buildings. Thus, investigative reports of ant infestations can be matched relative to landscape parameters on the outside of buildings and to the room characteristics at the site of the infestation. Identifying existing patterns can lead to insights regarding best landscaping design practices to minimize *L. humile* infestations.

2. Methods

2.1. Study Site. This study was conducted on the campus of UCLA. During the study period, the main campus had 94 buildings and structures, of which a number had chronic infestations of *L. humile* for multiple years. The campus landscaping is relatively uniform consisting primarily of

combinations of grass, ivy, shrubs, and several species of trees (mostly sweet gums, sycamores, eucalyptus, and several species of pine). The landscape is interspersed with solidly paved walkways that create gardens of various sizes adjacent to buildings (Figure 1). These paved areas all have heavy human foot traffic. Although it is likely that the ants at UCLA are a single unicolonial [3] population interconnected by trails and the movements of foragers, the pavement dividers create areas where ants cannot nest and are found only when travelling. Hence, gardens are the only islands of high-density nests from which infestations can radiate to adjacent buildings. Argentine ants can be found in every gardened area on the campus, and no other ant species are evident (Gilboa and Nonacs, pers. obs.). All gardens were regularly irrigated and soil moisture levels did not differ significantly across sites. For analyses, the predominant vegetation defining given gardens were combined into three inclusive categories: grass, low groundcover other than grass (e.g., ivy), and bushes or hedges. A significant food source for *L. humile* is honeydew from tended insects [3, 4]. We did not measure insect densities (other than ants) across vegetation types. Therefore, any differences across vegetation categories may be due to differences that are physical, biological, or both.

2.2. Tracking Infestation Patterns. We obtained the records of complaint calls to UCLA's central Facilities Management about ant infestations for the period of March 2002–August 2003. Across this time period, infestations were reported in 28 buildings on campus. After receiving a report, descriptive details of the infestation were collected at the site. This included the following: (1) the room location; (2) noting within the room whether water, food, or plants (i.e., potential *L. humile* nest sites) were present; (3) measuring the size of the closest garden outside the building to the room of the infestation. The intensity of the infestation (i.e., number of ants) was not recorded as UCLA facilities personnel had usually treated the ants prior to our arrival, by placing commercial bait traps inside the infested rooms. Access to some sites of infestation was either not available or the exact location was not specified in the complaint. In total, data from 42 rooms in 15 buildings were collected. For 14 of the buildings (Figure 1) the size (in m^2, using precise ARC-GIS maps available for the UCLA campus) of the closest garden to the infested rooms and the predominant vegetation type were calculated and recorded. For the remaining building, the infestation occurred over 100 m from the nearest garden. All buildings had numerous human occupants and were all either classroom, office, laboratory, administration, or some combination thereof. All appeared structurally sound and were regularly maintained by facility personnel. None were under construction or major renovation at the time of the study. There were no obvious differences across buildings in terms of potential entry points for ants.

Buildings that did not report infestations could not be assumed to be ant-free. Lack of complaints could also be due to higher tolerance for infestations of only a few ants by the building's occupants, or problems being handled at the site rather than being reported to central facilities. Furthermore, tolerance for ants appeared to vary across buildings (e.g., Business School occupants seemed more willing to complain about ants than occupants of Life Sciences buildings). Such human behavioral differences, however, do not affect the time series analyses because buildings act as their own controls. It is assumed that the tolerance level towards ants, *per se*, in a given room, in a given building is the same relative to the immediate weather conditions, the time of year, and the nature of the nearest outside garden. Therefore, changes in the numbers of complaints about ants reflect changes in ant and not human behavior.

The activity levels of ants in the gardens closest to infestations were measured by 10 buildings (of the 14 reported above), 2 of which were measured in both summer and winter months. Measurements took place in August-September (2003) and January–March (2004). Ant activity was estimated by the rate of removal of 50% solutions of sugar water from 9-10 vials placed in each garden under clay pots (to minimize evaporative loss). Vials initially contained approximately 22 g of solution. Control vials which did not allow ant access measured evaporative loss. The amount of solution taken was measured every day, and the vials were never completely emptied over this time period. Based on an estimate that an *L. humile* worker can take 0.3 mg per visit, the total number of ant visits from the amount of liquid removed in a day were calculated (see [6, 17] for details of methods). This method is recommended as accurate and the least time-consuming for monitoring comparative levels of Argentine ant activity [17]. Because the collected data were often not normally distributed, nonparametric statistics were mostly employed. Statview was used for all statistical analyses.

Weather data (e.g., daily temperature and rainfall) were used from an on-campus weather station operated by the National Oceanic and Atmospheric Agency.

3. Results

3.1. Factors Affecting Ant Activity. Measured mean ant activity was higher in outside gardens in hotter months (mean maximum temperature > 21°C) than in colder months (7234 versus 1936; Mann-Whitney U test, $Z = -2.038$, $P = 0.0415$), and in drier months (cumulative rainfall < 1 cm) than wetter months (11126 versus 2639; Mann-Whitney U test, $Z = -2.208$, $P = 0.0272$). However, neither the size of the garden (range: 116–1123 m^2) nor the predominant type of vegetation (bushes, grass, or ivy) had a significant effect on the number of visits to baits (Table 1). This suggests that whatever physical or biological characteristics differed across the vegetation types, they did not significantly affect *L. humile* activity patterns. These data were also subdivided according to whether a given month was hot or cold and dry or wet to identify specific effects of temperature and rainfall. In none of these subsets of data was there a significant relationship.

3.2. Factors Affecting Frequency of Infestation. Infestation rates, as number of calls complaining about ants, showed three peaks: in the summer months of both 2002 and 2003

UCLA campus map

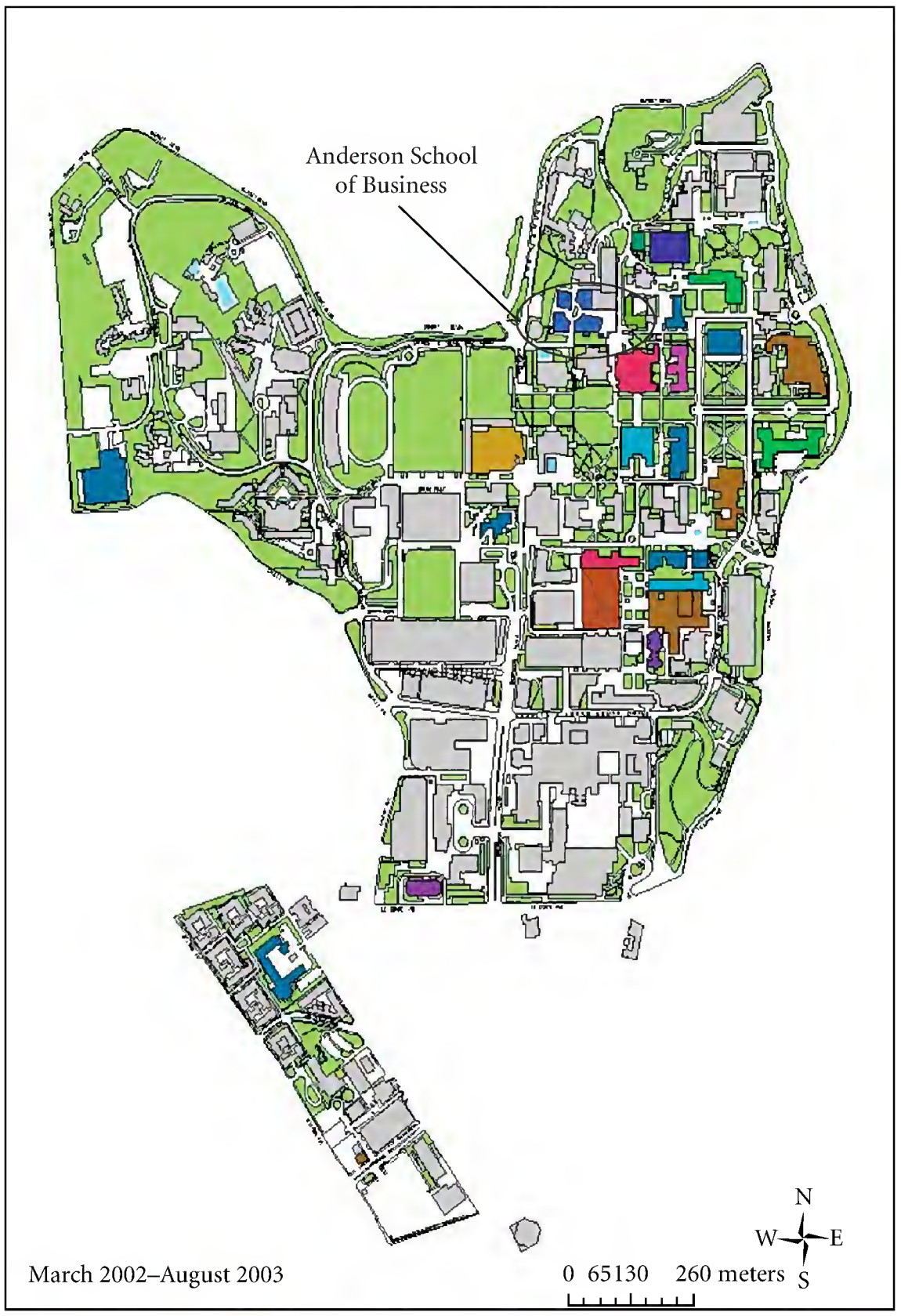

FIGURE 1: Map of UCLA *Linepithema humile* infestations in 2002-03. The Anderson School of Business is a complex of 4 structures, but only one of them reported all the infestations.

and in Jan/Feb of 2003 (Figure 2). In between these times there were relatively few ant infestations. Calls significantly increased with mean monthly maximum temperature ($y = -46.1 + 2.6x$; df = 1.16; $R^2 = 0.486$; $P = 0.0013$), but not in response to monthly rainfall ($y = 10.73 - 0.14x$; df = 1.16; $R^2 = 0.004$; NS) or number of days per month with measureable rainfall ($y = 12.19 - 0.76x$; df = 1.16; $R^2 = 0.024$; NS). Note that January of 2003 was much drier and hotter than normal (i.e., at the time, it was the warmest January on record for UCLA).

Neither ant density in gardens (assumed to correlate to number of visits to baits, range: 358–15684 sugar water loads removed) nor garden groundcover type, under any condition of monthly temperature or rainfall, were significant predictors of infestations in adjacent buildings (Table 1). Across all months, however, infestation frequency correlated significantly negatively with garden size (Table 1). Indeed, a small isolated garden in the midst of the business school complex (Figure 1) seemed the likely source for the many complaints, and ant trails entering the one infested building were directly observed upon inspection (S. Gilboa, per. obs.). These infestation data were also subdivided by mean monthly temperature and cumulative rainfall. In all cases, the same trend was exhibited: infestations were more likely next

to smaller gardens. The effect, however, was more strongly influenced by rainfall than by temperature (i.e., a statistically significant relationship is present only in wet months).

3.3. Factors Affecting Room Infestation. The main predictor of infestation for particular rooms was the presence of water. In the 42 infested rooms, 35 (74.4%) had either water sources (e.g., sinks, toilets, faucets, or standing water), potted plants, or a food source. Of these 35, 34 had a water source, 17 had plants, and 18 had food. Ten of the rooms had only a water source. One room had only a food source, and no rooms were infested that had only plants. Anecdotally, the occupants of the rooms often reported ants being primarily attracted to water and then mass recruiting if they also found food. The observed infestation pattern associating with water is unlikely to simply reflect a random sampling of rooms by ants. Most of the rooms in the infested buildings were either classrooms, meeting and seminar rooms, or offices (i.e., far fewer than 75% of the rooms in these UCLA buildings have a significant source of accessible water for ants).

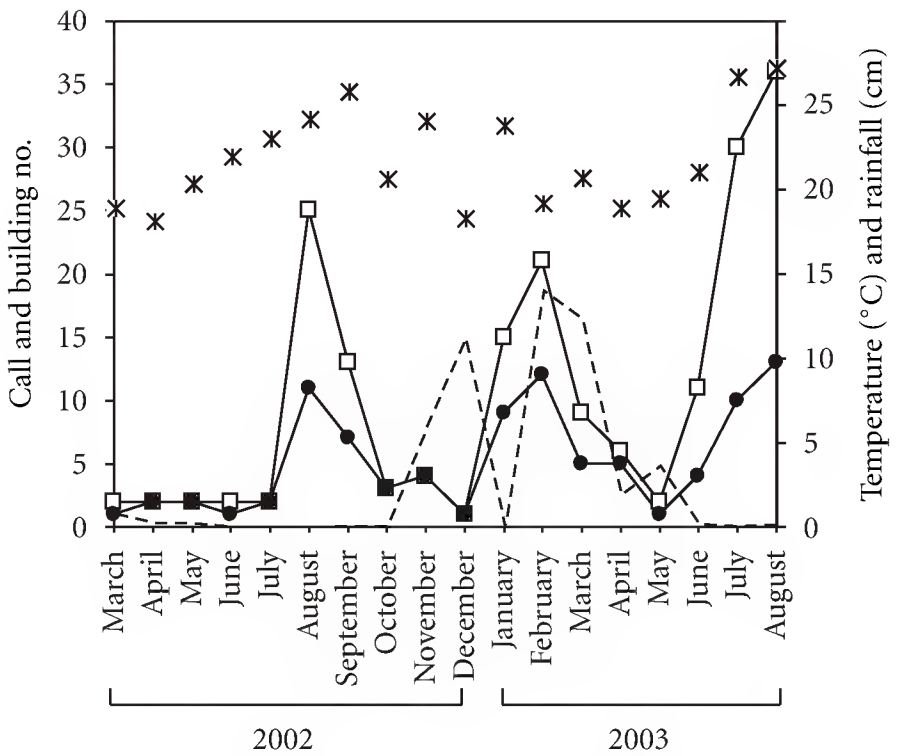

Figure 2: Month to month record of infestations and climate data at UCLA. Lines with open squares are the number of total calls; lines with closed circles are the number of buildings infested. The crosses are the mean maximum temperature for the month, and the dashed line is the cumulative monthly rainfall (both from the UCLA station of the National Environmental Satellite, Data, and Information Service).

4. Discussion

Argentine ant activity on the UCLA campus is similar to that reported elsewhere [18–20]: infestations are at a minimum from March through June which correlates with lowest points of worker numbers [21], worker activity thereafter peaks in the summer, and infestation intensity has a summer and a winter peak (Figure 2). These results differ from Gordon et al. [19] in finding that infestations positively correlate to temperature but are not predicted by rainfall. Northern California has both colder winters and heavier winter rains. Thus the higher incidence of winter infestations may be due to ants seeking warmer and drier nest sites. Interestingly, the one peak in infestation observed in our winter data correlated with a record-breaking January heat wave.

The strongest predictor of where infestations occur in buildings is the presence of a water source (although food and potential nesting sites may influence the number of invading ants and the likelihood of repeated infestations). Several other aspects of our data and findings by others support the hypothesis that water-seeking behavior is a prime factor for why *L. humile* enters buildings. In our study, the greatest number of infested buildings and complaint calls occurred during hotter and drier months when water-stress is more likely to be an issue. Several ecological studies have also found that soil moisture and water availability have the strongest effect on the distribution of *L. humile* nests and the species success as an invasive [8, 13, 20–25]. If forced to choose, *L. humile* will prefer nesting in areas of optimal humidity rather than be near food sources [23, 24]. Other factors, such as vegetation, appear to play indirect roles through their positive effects on soil moisture. A second study on the UCLA campus found that *L. humile* presence within structures can be significantly reduced by placement of water on the outside of those structures [26].

The data show no effect of local densities of foraging ants in gardens on infestation rates, despite a 40-fold difference in local ant activity. This is particularly interesting because it implies that the increase in summer infestations cannot be accounted for by the significantly greater numbers or activity levels of ants in particular gardens. One caveat to the results may be, however, that all our measured densities including the lowest are indicative of a large local population of ants that are available to infest buildings. This is a typical pattern in *L. humile* where local densities of foragers tend to be high and not correlated to soil dryness [12, 14, 18, 20, 25]. There was also no significant effect of plant species across gardens in terms of either affecting ant densities or likelihood of infestation. It might be that *L. humile* are insensitive to differences in UCLA plant communities because they receive relatively similar watering regimes. Whether or not the differing plant communities provided different amounts of food for *L. humile*, such as varying densities of honeydew-providing insects was unknown. However, to any extent that such differences existed, they did not significantly affect infestation patterns.

There was a significant effect of garden size, where surprisingly smaller gardens tended to be associated with increased likelihood of infestation. A potential explanation for this correlation may be that smaller gardens have less food (e.g., fewer homopterans, in total, to tend) and water to maintain the high density of ants. Given the large foraging range that *L. humile* colonies can exhibit [3], infestations may be the result of "normal" Argentine ant foraging ranges. The small garden effect appears to be particularly evident in wetter months, where reduced food availability might stimulate enlarging the foraged areas.

The attraction that Argentine ants have for suitably moist habitat suggests manipulating water availability can be useful for integrated pest management in urban situations.

Table 1: Summary statistics using Spearman rank correlations (Z-values) and Kruskal-Wallis tests (H values). For the Kruskal-Wallis tests b, g, and i refer to number of gardens with predominant groundcover of bushes, grass or ivy, respectively.

Garden size and ant density	Z-value	P value	n
All months	−0.377	NS	12
Hot months (>21°C)	0.252	NS	8
Cold months (<21°C)	−0.693	NS	4
Wet months (>1 cm)	−0.898	NS	8
Dry months (<1 cm)	1.386	NS	4
Garden type and ant density	H value	P value	b, g, i
All months	0.526	NS	3, 3, 6
Hot months (>21°C)	0.717	NS	1, 2, 5
Cold months (<21°C)	0.300	NS	2, 1, 1
Wet months (>1 cm)	0.125	NS	2, 2, 4
Dry months (<1 cm)	2.700	NS	1, 1, 2
Infestations and ant density	Z-value	P value	n
Hot months (>21°C)	−0.598	NS	8
Cold months (<21°C)	1.126	NS	4
Wet months (>1 cm)	1.606	NS	8
Dry months (<1 cm)	−0.693	NS	4
Infestations and garden type	H value	P value	b, g, i
All months	0.606	NS	3, 4, 7
Hot, dry months (>21°C, <1 cm)	0.366	NS	3, 4, 7
Cold, dry months (<21°C, <1 cm)	0.008	NS	3, 4, 7
Hot, wet months (>21°C, >1 cm)	0.733	NS	3, 4, 7
Cold, wet months (<21°C, <1 cm)	1.168	NS	3, 4, 7
Infestations and garden size	Z-value	P value	n
All months	−2.104	0.0354	12
Hot, dry months (>21°C, <1 cm)	−1.853	0.0638	12
Cold, dry months (<21°C, <1 cm)	−1.525	NS	12
Hot, wet months (>21°C, >1 cm)	−2.104	0.0354	12
Cold, wet months (<21°C, <1 cm)	−2.299	0.0215	12

Clearly within buildings access to water can be controlled [7]. Outside of buildings, planting drought-resistant, xeric gardens may keep larger nests away from structures [24]. If these are not options, then the reverse is also possible; provide easily accessible water to the ants particularly during times of hot, dry weather. If water is available on the outside and away from structures, this may keep ants from searching inside buildings. For example, using less than a liter of water per day, Enzmann et al. [26] significantly shifted the movement patterns of *L. humile* populations from the inside of structures to the outside. Such manipulations may be particularly useful if ants are present in gardens smaller than their normal foraging range. In conclusion, because trails of *L. humile* form connected networks [22, 27], this may present the opportunity to minimize ant problems through moving those networks with simple landscaping solutions.

Acknowledgments

The authors thank L. Lowe and J. Powazek from UCLA Facilities Management. This work was supported by UC-IPM Grant 02XA013 from the USDA. Brittany Enzmann and Jeff Thomas helped with the placement and collection of ant baits.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 806782, 7 pages
doi:10.1155/2012/806782

Research Article

Dynamics of Foraging and Recruitment Behavior in the Asian Subterranean Termite *Coptotermes gestroi* (Rhinotermitidae)

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Received 5 April 2012; Accepted 18 May 2012

Academic Editor: Russell Jurenka

The present study investigated the trail-following behavior of the subterranean termite *Coptotermes gestroi* (Wasmann Rhinotermitidae) under laboratory conditions. The results showed that workers were the first to initiate the exploration to the food source. When food was discovered they returned to the nest laying a trail for recruiting nestmates to the food source. In this situation, workers always traveled significantly faster when returning from the arenas. Both workers and soldiers were recruited to the food source; however, the soldier/worker proportion was higher during the first phase of the recruitment. When no food was available, the number of recruited nestmates and the speed on their way back to the nest were significantly lower. The results also showed that scout foragers always laid trail pheromones when entering into unknown territories, and that chemical signals found in the food could induce workers of *C. gestroi* to increase their travel speed.

1. Introduction

The dynamics of the foraging and recruitment process in social insects has been investigated in many species within the Isoptera and the Hymenoptera. While many ants and bees also rely on optical cues, foraging in termites is organized predominantly by chemical signals, such as pheromone trails laid on the substrate for orientation and recruitment. The sternal gland is the only reported source of trail pheromone in termites. Secretions of this gland are considered to function in the recruitment of nestmates to source disturbance within the nest. Termites may also use trail-following pheromones to colonize new food sources [1, 2].

Termite trail-following pheromones are composed of one or few compounds. Up to date, only 9 compounds have been identified as trail pheromones in several termite species. For the termite families in which chemical trail pheromones have been reported, Rhinotermitidae, Termitidae, and Kalotermitidae seem to use mainly neocembrene A, (*Z*,*Z*,*E*)-dodeca-3,6,8-trien-1-ol, (*Z*)-dodec-3-en-1-ol, and (Z,Z)-dodeca-3,6-dien-1-ol [3–9]. *Nasutitermes corniger* uses in addition to these two compounds, trinervitatriene [8]; Mastotermitidae uses (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol and Termopsidae uses (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol, tricosane, and 4,6-dimethyldodecanal; whereas *Glossotermes oculatus* (Serritermitidae) uses nonecadienone for trail following [10, 11]. Termites also seem to use secretions of the sternal gland as both attractants and orientation signals in their foraging trails [12, 13], but the details of the chemical communication system used by termites are less known than in the Hymenoptera. Nevertheless, behavioral evidence also suggests that additional compounds may act in species specificity and in the differentiation between exploring and foraging trails [14, 15]. Despite the fact that few components have been identified, behavioral evidence suggests that termite trail pheromones are multicomponent systems as shown for some ants [16]. Furthermore, qualitative or quantitative differences in pheromone trails laid by different castes have been suggested as cause for caste-specific polyethism during termite foraging and recruitment [17–19] and for differentiation between foraging and recruitment trails [20].

Subterranean termites (Rhinotermitidae) create ramified tunnel systems above or beneath the soil to locate their cellulosic food. Sometimes these tunnels, which range from tens to hundreds meters in length, connect multiple feeding

sites. The Asian subterranean termite *Coptotermes gestroi* (Rhinotermitidae) is a pest of great economic importance in urban areas of southeastern Brazil. This species was accidentally introduced from marine cargo, probably at the beginning of the 20th century and has rapidly colonized new areas throughout southern Brazil, increasing its economic impact [21]. As discussed elsewhere [22, 23], foragers of *C. gestroi* build several foraging tunnels at random. When the food is located, there is an increase in the number of workers and soldiers in the tunnels that connect the nest directly with the food (recruitment tunnels). At this time, foraging trails could be abandoned by the foragers. The aims of this study were to characterize foraging and recruitment trails on a behavioral level in *C. gestroi* (Wasmann). We examined the response toward two different types of trails, and caste-specific differences toward trails laid by workers or soldiers. Workers of *Reticulitermes santonensis* are able to discriminate trails that connect the nest to food sources from others [15]. Since (*Z*,*Z*,*E*)-dodeca-3,6,8-trien-1-ol is the only compound found in the trail pheromone of *R. santonensis* [24] and *C. gestroi* [25], we hypothesized that differences on the trail following behavior between foraging or recruitment trails could be due to differences in the concentration of the trail pheromone laid by the termites. Although termite trail communication has been studied for decades, detailed knowledge is only fragmentary. Such information would be useful in understanding the foraging behavior and food preference patterns of this species with the objective of improving a pest management strategy against this invasive pest termite.

2. Methods

2.1. Collection and Maintenance. Foraging workers and soldiers of *Coptotermes gestroi* (Rhinotermitidae) were collected from natural colonies in Rio Claro city, São Paulo State, Brazil (22°23′43″S, 47°32′39″W) . The termites were collected using traps of corrugated cardboard paper placed on the foraging territories of the colonies of each species. The traps were collected after one week and the individuals (workers and soldiers) were confined in 2.5 L glass containers filled with wet sand (at 10% moisture) and stored at 25°C, according to the laboratory maintenance protocols established for this species [19]. Food consisted of 3 × 2 × 1 cm blocks of *Pinus* sp. For bioassays on foraging and recruitment trails; 200 workers and 20 soldiers were transferred into a 500 ml plastic container (artificial nest) and acclimatized for one week until the beginning of the bioassays. Five colonies were used in this study.

2.2. Sternal Glands Extracts. Workers were cold anesthetized (−20°C) and placed in a ventral position on a Petri dish. The fifth sternite containing the sternal gland was gently removed under a stereomicroscope with the aid of microscissors. Samples of 10 glands of workers were transferred to vials and extracted in 100 μL of *n*-hexane for 24 h at room temperature. Preliminary tests showed that a polar solvent such as methanol did not extract compounds that elicited trail following behavior in *C. gestroi*. The material was stored at −20°C until used for the bioassays. Due to unavoidable contamination with the over-developed frontal gland of the soldiers of this species, the sternal glands of this caste were not used in this study.

2.3. Activity of Sternal Gland Extracts. Activity was evaluated using a Y-choice assay on a 10 cm diameter filter-paper with a 120° angle between each branch. An artificial trail of gland extract (1 μL of extract per 1 cm of trail) was deposited at the base of the Y (3 cm) and on one of the branches (7 cm). In the same way, on the Y base and on the other branch we deposited a hexane extract as control. The distance covered by each individual (worker or soldier) was recorded using the following concentrations of the sternal gland extracts: 0.001 glands/μL (gl/μL), 0.005 gl/μL, 0.01 gl/μL, and 0.1 gl/μL. The maximal response was 10 cm, and the threshold concentration was defined as a trail followed longer than 3 cm. The individuals were previously confined into a device that allowed the exit of one individual at a time. For each replicate, a new filter paper was used in order to prevent trail reinforcement. Thirty replicates were made for each caste and concentration. Data were analyzed by a *t*-test to compare the activity between gland extracts and the control. An ANOVA was used to compare the mean distances covered by both workers and soldiers in response to gland and control extracts. Multiple comparisons among the means were performed using the Tukey HSD test, $P < 0.05$ [26]. All bioassays were made under red-light illumination at constant temperature and relative humidity (25°C, 70% RH).

2.4. Activity and Preference for Foraging and Recruitment Trails. Trail-following behavior was investigated in a setup connected to the above mentioned artificial nest. Termites had access to experimental arenas (200 ml plastic containers) via a glass tube (length, 8 cm; ID, 0.5 cm). Foraging trails consisted of empty arenas. On the other hand, recruitment trails were investigated in the same way but connecting the end of the glass tube to an arena containing blocks of pine wood as a food source. In both situations, we let a single worker lay a trail in the glass tube returning from arenas either with or without food. Access to the glass tube could be opened and closed with a slide in which a hole of 0.5 cm was drilled. The travel speed of the first worker on its way forth and back was evaluated. After trail marking, one worker was allowed to travel through the glass tubes, and its travel speed was evaluated as mentioned before. The total number of termites following both types of trails, offered within 30 min after opening the slide, was also recorded. After each single replicate, the tube was removed and replaced by a new one. Trail-marked tubes were used in the preference bioassays. The experiment was replicated 20 times for each trail type. Data were analyzed by Mann-Whitney *U* test (comparison between foraging and recruitment trails) and Wilcoxon matched-pairs test (comparison of the ways back and forth for each trail type) [26]. In order to determine the persistence of both foraging and recruitment trails, the experiments were also conducted with test tubes marked by the termites 24 h before.

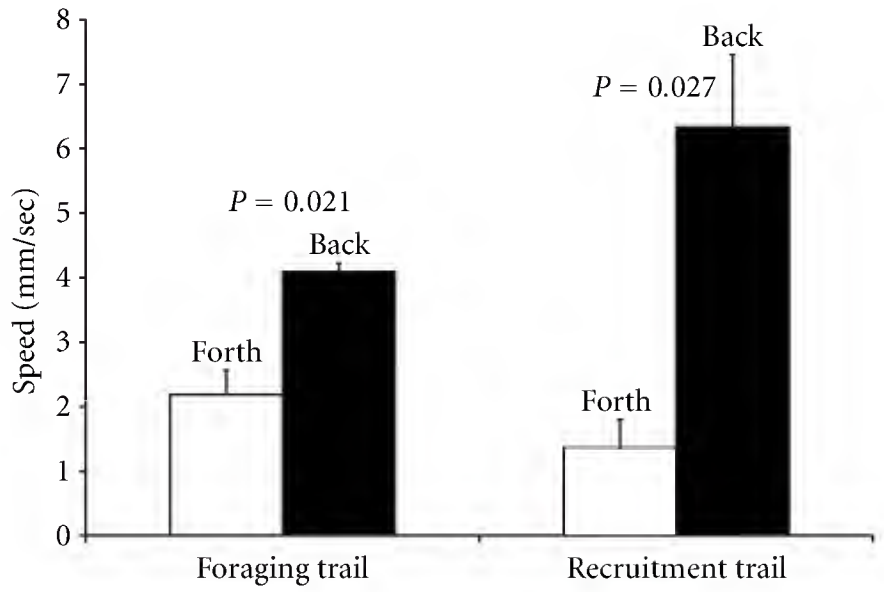

FIGURE 1: Mean travel speed (±SD) by trail-laying workers of *Coptotermes gestroi* in a glass tube connected to empty arenas (foraging trail) or wood (recruitment trail). $N = 20$; P values above bars correspond to the Wilcoxon matched-pairs test ($P < 0.05$) for differences between forth and back speed.

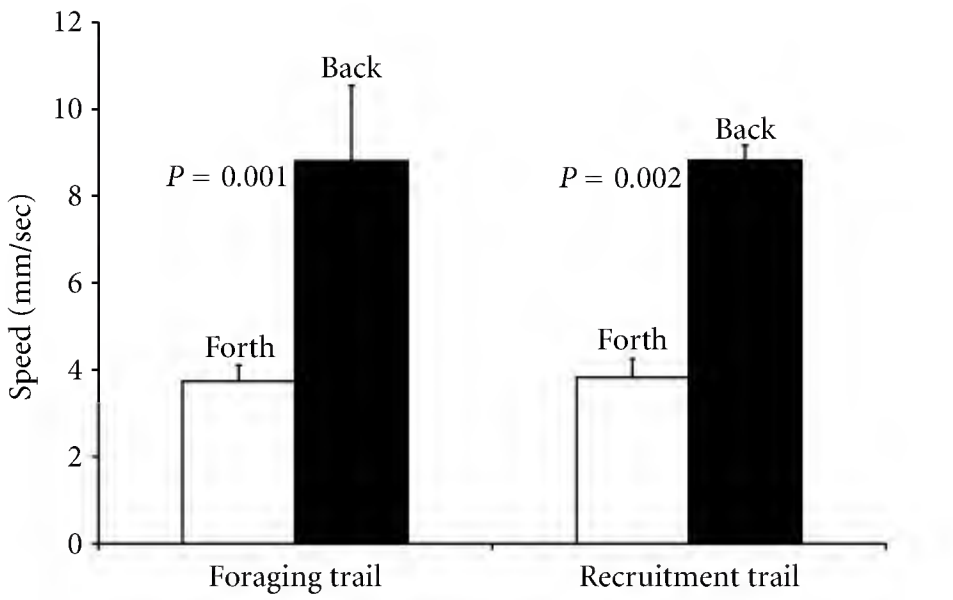

FIGURE 2: Mean travel speed (±SD) of the first worker following foraging or recruitment trails. $N = 20$; P values above bars correspond to the Wilcoxon matched-pairs test ($P < 0.05$) for differences between forth and back speed.

To examine the preference of termites to different trail types, the workers were able to deposit a foraging or recruitment trail for 30 min as described above. Both control and test tubes with the trail were then attached simultaneously to the artificial nest in the setup described above. Controls consisted of clean glass tubes. The choice behavior of the first worker following the offered trail was recorded. This experiment was replicated 30 times for each trail type. To evaluate the persistence of trails laid by the termites, we used test tubes marked by the termites one week and one month before the bioassays. The results were analyzed using a binomial Z test [27]. All bioassays were made under red-light illumination at constant temperature and relative humidity (25°C, 70% RH).

3. Results

The worker caste of *C. gestroi* was the first to initiate the exploration towards the new food source. The present data showed that they initiated the exploration in 73% of the assays ($\chi^2 = 24.30$; df = 1; $P < 0.001$; Yates correction), independently of the type of foraging situation, that is, with or without food ($\chi^2 = 0.67$; df = 1; $P < 0.414$). Afterwards, workers returned to the artificial nest, abdomen pressed to the substrate, chemically marking the initial exploratory trail. The time at which scout workers traveled to the arenas in the glass tube was determined for foraging and recruitment trails. In both situations, workers traveled significantly faster when returning from the arenas (Figure 1). But if wood was offered, travel speed returning to the nest was significantly higher than in the absence of food ($P = 0.047$, Mann-Whitney U test) (Figure 1). This behavior started the recruitment phase. When workers detected both types of trails and followed them. In our bioassays, the first recruited worker traveled significantly faster when returning from the arenas ($P < 0.05$; Wilcoxon matched-pairs test). However, significant differences of the travel speed on the way back and forth with or without food were not detected ($P > 0.05$, Mann-Whitney U test) (Figure 2).

After 20 min, a significantly greater number of workers arrived to the food source (recruitment trail) ($P < 0.05$; Mann-Whitney test). On this type of trail, the maximum number following within 30 min rose to above 23 (Figure 3(a)). The number of workers recruited in the foraging trails stabilized after 10 min of starting the bioassay. Eventually, the termites would build a covered gallery inside the glass tube, leading from the artificial nest containing the termites to the food. Soldiers were also observed following the trails and their number increased significantly after 15 min in recruitment trails ($P < 0.05$; Mann-Whitney U test) (Figure 3(b)). During the first phase of the trail-following behavior (5–20 min), the soldier/worker proportion was approximately 1 : 1 in recruitment trails. In foraging trails, this proportion was significant lower ($P < 0.05$; Mann-Whitney U test). But the soldier/worker proportion on the recruitment trail decreased after 15 min, as the number of recruited workers increased (Figure 3(c)). The total number of termites participating in the recruitment process represented a small proportion (about 10%) of the total number of termites confined in the artificial nests.

The preference of both foraging and recruitment trails over control trails indicates that foragers of *C. gestroi* always laid trails when exploring for food sources, even in the absence of food. When both types of trails were tested simultaneously, workers significantly preferred recruitment trails over foraging trails, indicating that the signal laid by the termites is different between these types of trails (Table 1). Recruitment trails laid by *C. gestroi* showed a high persistence in the glass tubes. When we offered a trail marked one week and one month before the choice bioassays, workers also showed a strong preference for their own trails over the control (Table 1).

The trail-following activity elicited by sternal gland extracts was higher than those of the control extracts in *C. gestroi* ($t = 18.43$, df = 1, $P < 0.0001$; t-test). The residual activity of the control extracts was probably

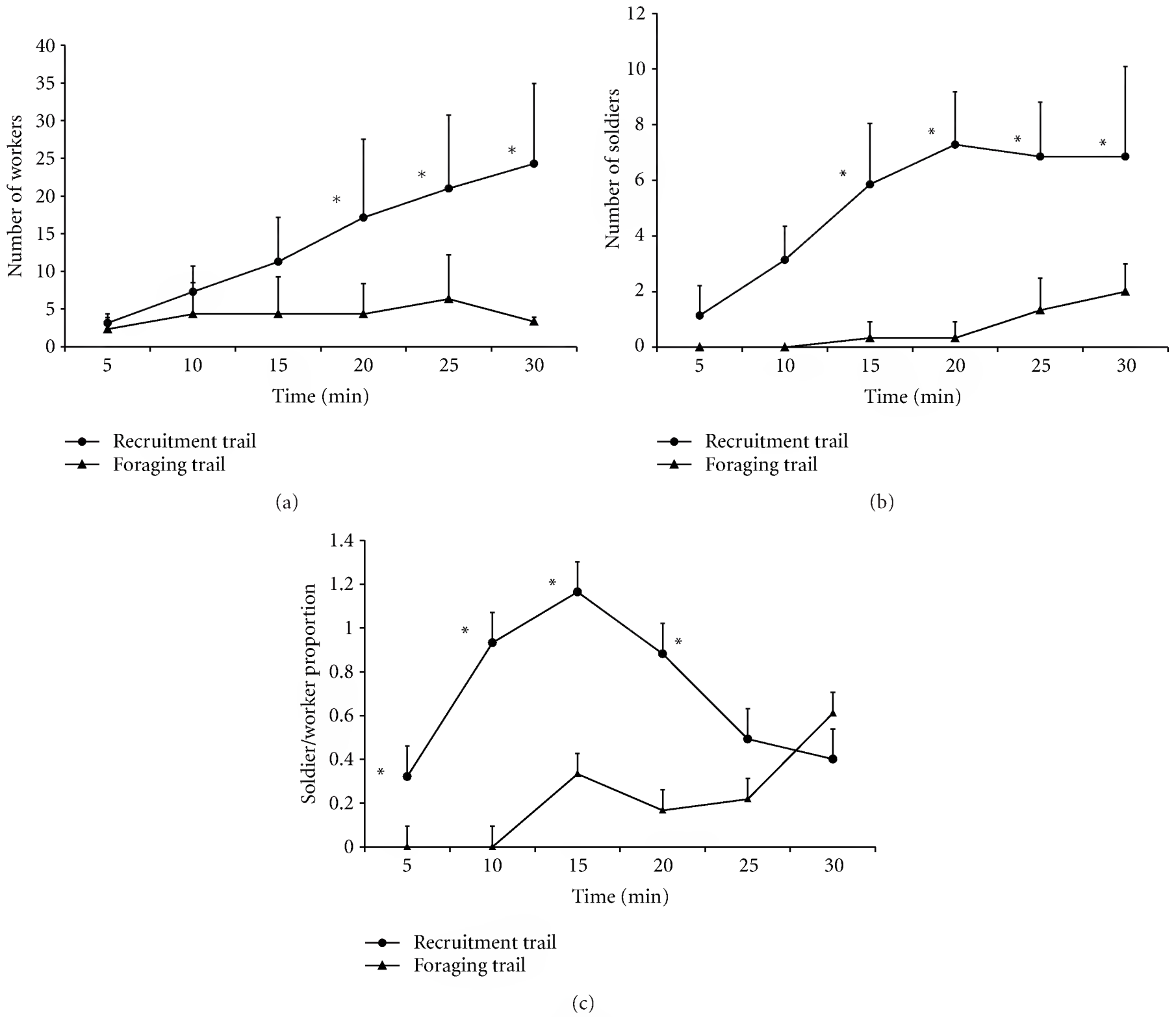

FIGURE 3: Mean number (±SD) of workers (a), soldiers (b), and proportion of soldiers and workers (c) of *Coptotermes gestroi* following a foraging or recruitment trail within 30 min. $N = 20$. Asterisks indicate significant differences between types of trails ($P < 0.05$; Mann-Whitney test)

TABLE 1: Trail-following preference* of *Coptotermes gestroi* workers on foraging and recruitment trails.

Time after trail marking	Foraging trail		Recruitment trail		P
	Marked	Unmarked	Marked	Unmarked	
30 min	20	8			0.002
30 min			24	6	<0.0001
30 min	6		24		<0.0001
1 week			18	8	0.009
1 month			20	5	<0.0001

* The results were analyzed using the binomial test ($P < 0.05$); $N = 30$.

due to contamination of trail pheromones after removal of the sternal glands. Trail-following behavior was affected by the concentration of sternal gland extracts. Activity of both workers and soldiers increased significantly with increasing concentrations of the sternal gland extracts (workers: $F = 201.59$; df $= 3$; $P < 0.0001$. Soldiers: $F = 174.75$; df $= 3$; $P < 0.0001$; ANOVA). Both castes registered their maximum activity at the concentration of 0.01 gl/μL, and their activity threshold was observed at 0.001 gl/μL. Concentrations higher than 0.01 gl/μL clearly reduced the trail-following activity of the individuals (Figure 4).

4. Discussion

Foraging behavior in termites is a collective process mediated by semiochemicals produced by exocrine glands of both workers and soldiers. The sternal gland is the only known source of trail pheromones in termites, and secretions of this gland are considered to function in the recruitment of nestmates from nest to feeding sites. *Coptotermes gestroi* and other subterranean termites have a similar foraging behavior in the presence of a new food source. The behavior of termite workers when the scouts look for a new food source was described by Arab et al. [19] for *C. gestroi*, Reinhard

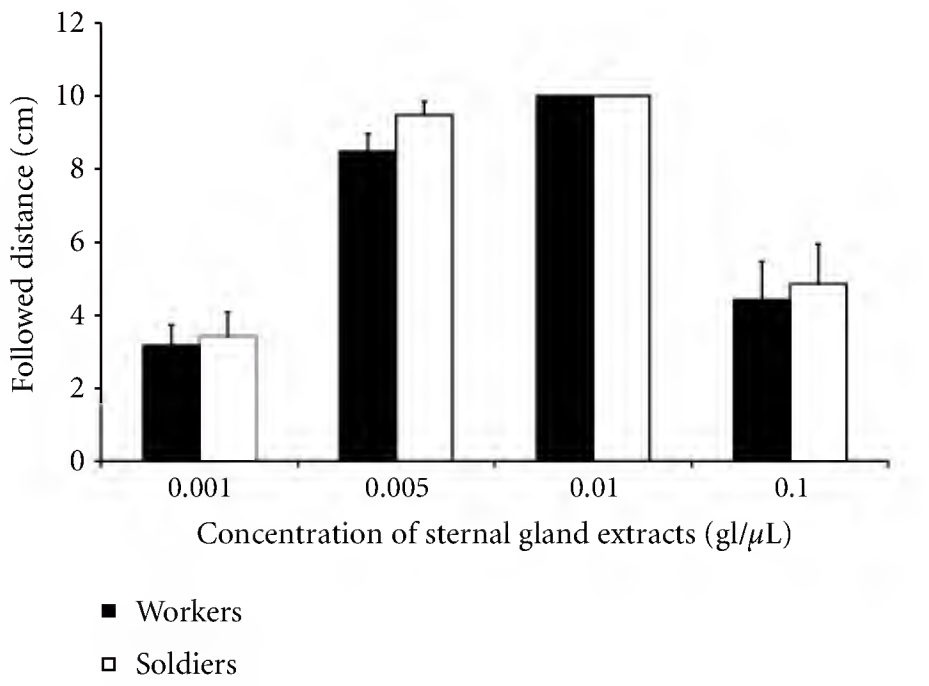

FIGURE 4: Termite activity elicited by sternal gland extracts of *Coptotermes gestroi*. Values indicate mean followed distance (±SD) by workers and soldiers. $N = 30$.

and Kaib [15] for *Reticulitermes santonensis*, and Traniello and Busher [18] for *N. costalis*. The results of these studies and the present one showed that: (1) both castes (mainly workers) initiate the exploration to new food sources; (2) trail pheromone is laid on the substrate; (3) both castes are recruited from the nest; (4) the number of individuals recruited depends on the presence of food; (5) soldier/worker proportion is higher at the first stage of the recruitment process.

The present data indicate behavioral differences in the way that termites react to the different trail types. Travel speed of *C. gestroi* scout workers was always higher on the way back to the nest. This probably is due to the fact that scout foragers always laid trail pheromones when entering into unknown territory, and thus are able to follow their own trail back to the nest. These results also indicate that the subsequent discovery of a food source by workers also increases their travel speed. When food is discovered, the travel speed on the way back to the nest was higher in recruitment trails as compared to the foraging situation (without food). According to Clèment et al. [28] and Reinhard et al. [29], wood volatiles can induce trail-following behavior in subterranean termites. However, in the subterranean termites, the presence of food does not seem to elicit the building of underground tunnels, instead, workers of *C. gestroi* build tunnels randomly in their foraging territories [30, 31]. Probably, chemical signals found in the food or other factors, such as chewing behavior [32] or the presence of a full gut in the returning termites could induce workers of *C. gestroi* to increase their travel speed, therefore, maximizing the foraging performance when food is discovered.

Recruitment trails of *C. gestroi* were more preferred and attractive to workers than foraging trails. They were followed by more termites than foraging trails. A higher attraction of recruitment trails compared to foraging trails has also been described for *Trinervitermes bettonianus* (Termitidae), *R. flavipes* (Rhinotermitidae), and *Nasutitermes costalis* (Termitidae) [12, 20, 33]. The difference between the two types of trails may be connected to qualitative or quantitative differences, that is, termites could either release more pheromone to lay a recruitment trail or release additional pheromone components. Qualitative differences of the trail pheromone can be more expected in the Termitidae, since the trail pheromone identified in some species of this family is a blend constituted by at least two components, which can be modulated to elicit different responses on nestmates. Conversely, only ((*Z*,*Z*,*E*)-dodeca-3,6,8-trien-1-ol) has been identified in the trail pheromone of workers of *R. santonensis* and *C. gestroi*, suggesting that preference for trail types in these species could be modulated by differences of the concentration of the trail pheromone laid by nestmates. Further components in the trail pheromone of these species have not yet been identified. Workers of *R. santonensis* termites pressed their abdomen onto the ground in a dotted way in foraging trails. In contrast, for recruitment trails the abdomen was dragged over the surface, resulting in a continuous trail. During dragging, more sternal gland secretion could be applied [1, 15]. Direct observations were unable to detect differences in trail pheromone deposition; however, activity bioassays showed that higher concentrations of sternal gland extracts elicited a higher activity in both workers and soldiers of *C. gestroi,* as demonstrated by Grace et al. [34] for *R. hesperus*.

Long-lasting, low-volatility trail pheromones could be useful for trail orientation when termites forage in tunnels built underground or on the surface [25] and references therein, and trail information could be modulated by the concentration of the trail pheromone. However, (*Z*,*Z*,*E*)-dodeca-3,6,8-trien-1-ol is a volatile and unstable molecule [8] and cannot remain active for long periods. Since the present bioassays showed that recruitment trails remain active after one month, it is possible that other signals play a role in modulating this type of communication in *C. gestroi*. Many termite species add feces, saliva, and other secretions to the trail. This could explain why foraging trails can be reused after several years in some termite species [20].

Caste-specific polyethism during foraging and recruitment has been observed in some termite species [18]. Scout individuals are specialized in exploring for food sources and recruiting nestmates by laying chemical trails [15]. The present results for *C. gestroi* show that workers initiate the exploration to new food sources. Then, both soldiers and workers are recruited; however, soldier/worker proportion was higher at the first stage of the recruitment. This caste-specific difference in recruitment behavior has been observed in other termites [15] and corresponds to the different roles of the two castes during the search for food [30]. In *C. gestroi*, workers initiate foraging most of the times, and the soldier/worker proportion remained high during the first phase of the foraging (5–20 min). This behavior could be associated with the defensive role of soldiers in the exploration to an unknown territory and was more evident in the presence of food (recruitment trails), thus suggesting that the trail pheromone could also modulate the defensive behavior of soldiers in colonies of this species.

The comparison of this research with other similar studies provided a better understanding of how a termite

colony organizes the process of foraging and recruitment. Recently, detailed research on the chemical composition of the trail pheromone of several species has been conducted (reviewed in [35, 36]). However, behavioral bioassays have not been performed in parallel to verify the presence of additional signals in the foraging behavior of termites. Future studies involving foraging bioassays will be necessary in order to expand the understanding of caste-specific roles in the trail-following behavior of termites.

Acknowledgments

The authors thank FAPESP (*Fundação de Amparo à Pesquisa no Estado de São Paulo*), PhD Grant 00/11451-3 and CNPq for financial support.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 145930, 6 pages
doi:10.1155/2012/145930

Research Article

Flight Dynamics and Abundance of *Ips sexdentatus* (Coleoptera: Curculionidae: Scolytinae) in Different Sawmills from Northern Spain: Differences between Local *Pinus radiata* (Pinales: Pinaceae) and Southern France Incoming *P. pinaster* Timber

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Received 12 April 2012; Accepted 22 May 2012

Academic Editor: Kleber Del-Claro

In January 2009, the windstorm "Klaus" struck the southern part of France, affecting 37.9 million m^3 of maritime pine *Pinus pinaster* Aiton (Pinales: Pinaceae). This breeding plant material favored the outbreak of *Ips sexdentatus* (Börner) (Coleoptera: Curculionidae: Scolytinae). As much of this timber is imported to the Basque Country (northern Spain), a potential risk to conifer stands is generated, due to the emergence of the incoming beetles. Thus, flight dynamics and beetle abundance were compared in different sawmills, according to the timber species (either local *P. radiata* D. Don or imported *P. pinaster*). A maximum flight peak of *I. sexdentatus* was observed in mid-June in *P. pinaster* importing sawmills, whereas a second lighter peak occurred in September. In contrast, only a maximum peak in mid-June was observed in *P. radiata* inhabiting beetles, being significantly smaller than in local *P. pinaster* trading sawmills. In addition, significant differences were found between imported *P. pinaster* and *P. radiata* regarding the number of insects beneath the bark. The development of IPM strategies for controlling *I. sexdentatus* populations is recommended, due to the insect abundance found in *P. pinaster* imported timber.

1. Introduction

Bark beetles (Coleoptera: Curculionidae: Scolytinae) are an insect group that contains at least 6,000 species from 181 genera around the world [1]. Bark beetles are considered as important agents of forest succession and initiate the sequence of nutrient cycling in infested tree material [2]. However, it is well known that some species are among the most destructive insects of coniferous forests, representing a continuous threat [1, 3]. Although bark beetles tend to colonize dead or weakened trees, it is well reported that some species can attack healthy trees under epidemic conditions. Frequently, improper forestry management or adverse abiotic and climatic conditions (e.g., storms, fires, and droughts) act as precursors by providing breeding substrate that unleashes population outbreaks for these bark beetles species [4–6]. For instance, the storms "Vivian/Wiebke" in February/March 1990 and "Lothar" in December 1999 triggered the propagation of *Ips typographus* (L.) in Centre Europe [7]. Recently, "Klaus" named windstorm affected 37.9 million m^3 of maritime pine *Pinus pinaster* Aiton (Pinales: Pinaceae) in Aquitaine (southern France) during January 2009 [8]. As a consequence a great amount of windthrown timber was left as suitable breeding material for the six-toothed beetle *I. sexdentatus* (Börner) Figure 1. Despite its preference for weak, decaying or dead trees, the six-toothed beetle can attack healthy trees under outbreak conditions. Much of this timber from Landes region is imported to many sawmill and timber-processing industries located at the Basque Country (northern Spain), due to its low cost. The long-time storage of such infested logs could put into risk the local forestry management, since new emerging *I. sexdentatus* would disperse beyond sawmills and attack the adjacent Monterey pine (*P. radiata* D. Don) stands,

(a)

(b)

(c)

FIGURE 1: *Ips sexdentatus* (Börner) (Coleoptera: Curculionidae: Scolytinae), lateral (a) and dorsal views (b), and detail of the elytral declivity of male (left) and female (right). Note the fusion at the base of the 3rd and 4th teeth in male (white arrow).

which is the most common tree species planted in the Basque Country [9].

Ips sexdentatus is a Palearctic species distributed throughout Europe which is capable of breeding in many coniferous genera, including *Pinus* L., *Picea* A. Dietr. (Pinaceae), *Larix* Mill. (Pinaceae), and *Abies* Mill. (Pinaceae) [10, 11]. Concerning the Basque Country, it has been trapped in both *P. radiata* and *P. sylvestris* L. stands [12]. It is associated with several species of ophiostomatoid fungi (Sordariomycetes: Ophiostomatales) [13, 14], which are involved in many tree diseases and sapstain [15]. Not only with blue-staining fungi, but also the association with the fungus *Fusarium circinatum* Niremberg and O'Donnell (= *F. subglutinans* f. sp. *pini* Correll et al. (Hypocreales: Nectriaceae), causal agent of the pitch canker disease, has been detected in *P. radiata* inhabiting populations in the Basque Country [16].

Thus, the aim of the current work was to determine the flight dynamics of *I. sexdentatus* in different sawmills from the Basque Country, according to different timber species (either *P. radiata* or imported *P. pinaster*). Secondly, in order to evaluate the infestation level of maritime pine, the density of beetles was evaluated, through direct observation on debarked logs. These primary objectives would allow inferring the significance and risk of importing maritime pine to the Basque Country.

TABLE 1: Sampling sawmills located at the Basque Country (northern Spain). *Pinus* L. species (Pinales: Pinaceae) is also indicated within each row.

Locality	Province	Latitude and longitude	Timber
Amezketa	Guipuzcoa	43° 02′ N, 02° 04′ W	*P. pinaster* Aiton
Tolosa	Guipuzcoa	43° 07′ N, 02° 04′ W	*P. pinaster*
Aia	Guipuzcoa	43° 15′ N, 02° 09′ W	*P. pinaster*
Berrobi	Guipuzcoa	43° 08′ N, 02° 01′ W	*P. radiata* D. Don
Zalla	Biscay	43° 12′ N, 03° 08′ W	*P. radiata*
Legutiano	Alava	42° 58′ N, 02° 38′ W	*P. radiata*

2. Material and Methods

Monitoring trapping took place from 1st April to 31th October 2011. Six different commercial sawmills were chosen. Three of them use *P. radiata* planted in the Basque Country as primary resource, whereas the other three import maritime pine timber from Landes region (southwestern France). The locations of sampling sites are provided in Table 1.

Two eight-unit Lindgren multiple funnel traps (Econex S.L., Murcia, Spain) were placed in each sawmill. Each trap was hung with the top of the trap at 2 m above the ground and the distance between traps was at least 50 m. One trap was unbaited, as a blank control, whereas the other trap was baited with a synthetic *I. sexdentatus*-specific pheromone (a mixture of ipsdienol (212.9 mg), *cis*-verbenol (60.8 mg), and ipsenol (13.6 mg), SEDQ, Barcelona, Spain). Baits were replaced every two months. Fifty mL of propylene glycol were added to each trap cup to kill and preserve captured insects. Not only *I. sexdentatus*, but also other bark beetles species and other accidentally trapped beetles were collected. Samples were removed every fifteen days and taken to the laboratory. Voucher specimens have been deposited at the Entomology Collection of the NEIKER-Basque Institute for Agricultural Research and Development, Arkaute, Basque Country, Spain.

In order to determine what *Pinus* species showed the largest density of *I. sexdentatus*, sections of 70 cm × 30 cm of seven randomly chosen logs (from both *P. radiata* and *P. pinaster*) were peeled off every week from 2nd May to 31th July in each sawmill. Debarking was made with the aid of a chisel. All *I. sexdentatus* present in the galleries beneath the bark were collected. The number of galleries was also recorded.

Data of mean catches of flying beetles caught in baited traps were subjected to a two-way ANOVA analysis (with pine species and date considered as factors). Subsequent Tukey *post-hoc* tests at a significance level of $\alpha = 0.05$

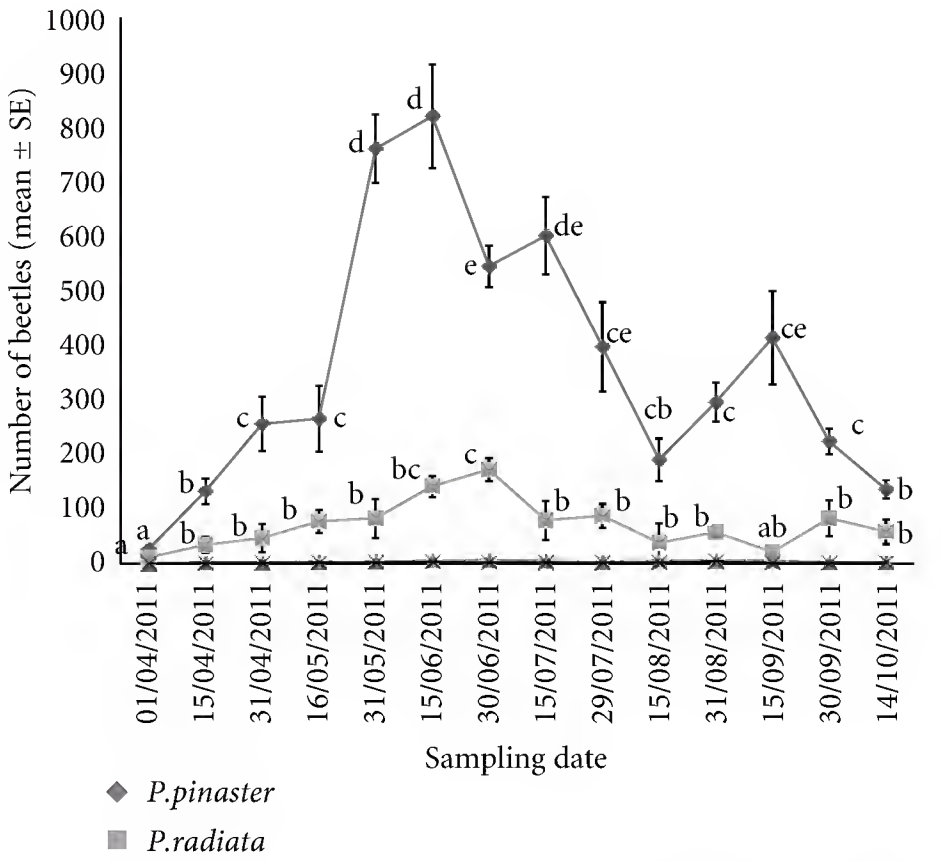

FIGURE 2: Number (mean ± SE) of *Ips sexdentatus* (Börner) (Coleoptera: Curculionidae: Scolytinae) captured in *Pinus pinaster* Aiton (Pinales: Pinaceae) (dark grey) and *P. radiata* D. Don (light grey) sawmills from 1 April to 14 October 2011. Dates within each *Pinus* L. species with different letters are significantly different at a significance level of $\alpha = 0.05$. Control catches in both cases were insignificant to perform any statistical analysis.

were applied to compare mean catches between dates within each *Pinus* species. Concerning density data, Student's *t*-test was used to compare mean number of galleries and beetle collections in different *Pinus* species for each month. A square root transformation was used to normalize the data and correct the heteroscedasticity. All the analyses were performed with the statistical software SPSS 2004 SYSTAT statistical package (version 13.0, SPSS, Chicago).

3. Results

A total of 15,184 specimens of *I. sexdentatus* were trapped in *P. pinaster* importing sawmills, whereas 2,774 were captured in *P. radiata* sawmills. As expected, pheromone-baited traps caught significant more insects in *P. pinaster* sawmills when compared with captures in *P. radiata* sawmills ($F = 108.927$, df = 1, $P < 0.001$). An interaction between sampling dates and *Pinus* species was found ($F = 7.2440$, df = 13, $P < 0.001$). A maximum flight peak was observed from the end of May to middle June for maritime pine, whereas a slighter peak occurred on September (Figure 2). Regarding *P. radiata* sawmills, a significant peak was observed only at the end of June. No statistical differences were observed in catches of other accidentally trapped insects.

Significant differences were found between the mean number of beetles and galleries under the bark during the three months. Maritime pine sections showed significant more galleries (May: $t = 4.152$, df = 12, $P = 0.002$; June: $t = 5.928$, df = 12, $P < 0.001$; July: $t = 5.063$, df = 12, $P < 0.001$) (Figure 3(a)) and beetles (May: $t = 9.367$, df = 12, $P < 0.001$; June: $t = 8.538$, df = 12, $P < 0.001$; July: $t = 7.900$, df = 12, $P < 0.001$) (Figure 3(b)) than in local *P. radiata*.

In addition, many other bark and ambrosia beetles species were accidentally captured in pheromone-baited traps. Table 2 details the different bark and ambrosia beetles caught per locality, along with other xylophagous species (Coleoptera: Cerambycidae) and bark beetle predators (Coleoptera: Cleridae).

4. Discussion

Current work demonstrates that maritime pine timber imported from France to commercial sawmills is highly infested compared to *P. radiata* timber, according to observed differences in the amount of insects caught in both field trapping and log debarking.

The six-toothed beetle has two generations per year, with adult flight periods from April to May and July to August. However, *I. sexdentatus* can undergo a third generation in Mediterranean regions of Europe [17]. Our results are consistent with other studies. Similar maximum flight peaks have been observed in *Picea orientalis* (L.) Link (in Turkey) and *Pinus sylvestris* (in Romania) stands [18, 19]. In contrast, *I. sexdentatus* showed three different peak flights in *P. pinaster* stands at the province of Leon (northern Spain), with the maximum peak occurring in September [20]. It has been suggested that this latter increase might be due to a strong increasing of the population during that season or a seasonal pheromone production, as it occurs in *I. pini* (Say) [21].

Ips sexdentatus is a polygamous species in which male is the pioneer sex which initiates the host seeking process. Afterwards, up to 2–5 females join each male within the gallery systems [10]. Galleries are star shaped, with a central nuptial chamber built by the male and in which mating occurs. Females bore egg galleries, which radiate outwards from the nuptial chamber. All the observed galleries in the current study had more than two arms.

Among accidentally trapped bark beetles species, it is worth noting the find of a female exemplar of the small spruce bark beetle *Polygraphus poligraphus* (L.), which would represent the first record for the Iberian Peninsula. *Polygraphus poligraphus* inhabits *Picea abies* (L.) H. Karst. and *P. obovata* Ledeb. [11], rarely breeding in *Pinus sylvestris* and *P. strobus* L. [10, 11, 22]. This unique specimen was trapped in the sawmill located at Berrobi, in which *P. radiata* timber is used. In addition, its distribution area is supposed to extend from Central Europe to Northern Europe and Siberia [11], being absent in the Mediterranean region [10]. Thus, the presence of this insect in the sampling area should be clearly stated.

Moreover, two species of *Monochamus* Dejean (Coleoptera: Cerambycidae) were also trapped, mainly in two *P. pinaster* trading sawmills: *M. sutor* (L.) and *M. galloprovincialis* (Olivier). The latter shows special relevance, as it is known to be the vector of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle (Aphelenchida, Parasitaphelenchidae), causal agent of the pine wilt

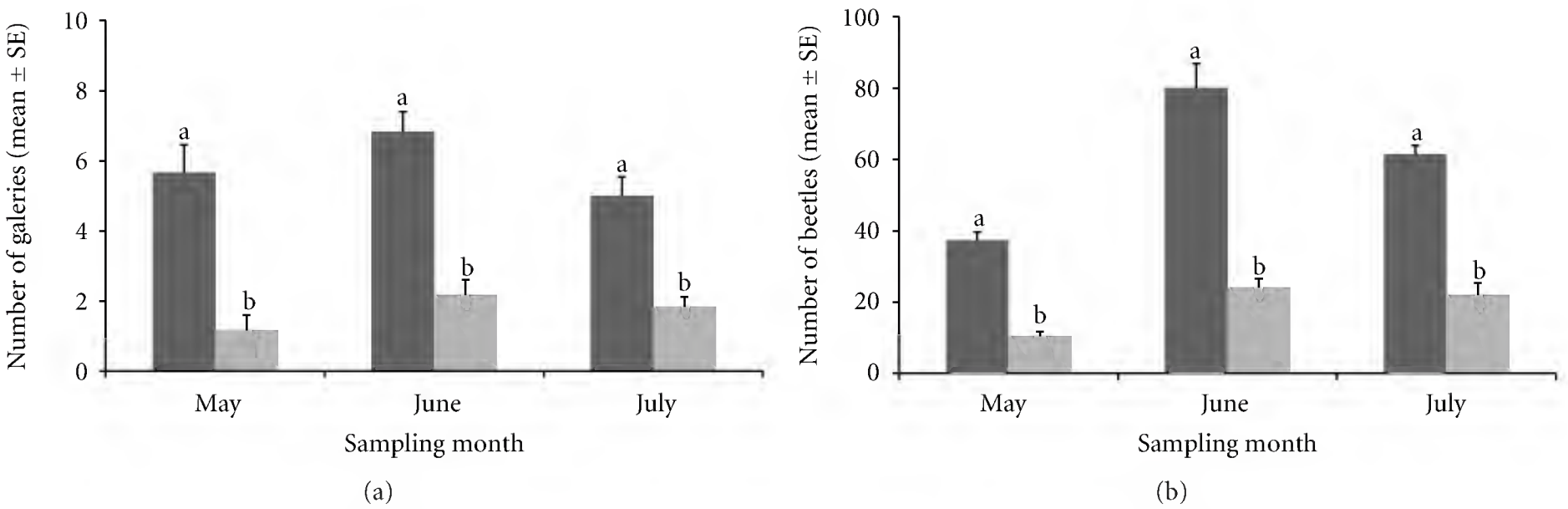

FIGURE 3: Number (mean ± SE) of (a) galleries and (b) *Ips sexdentatus* (Börner) (Coleoptera: Curculionidae: Scolytinae) found under the bark of *Pinus pinaster* Aiton (Pinales: Pinaceae) (dark grey) and *Pinus radiata* D. Don (Pinales: Pinaceae) (light grey) logs from May to July ($n = 28$). Means within each month with different letters are significantly different at a significance level of $\alpha = 0.05$.

TABLE 2: Total number of accidentally trapped species of bark and ambrosia beetles (Curculionidae: Scolytinae), cerambycid (Cerambycidae) and checkered beetles (Cleridae). Species within family/subfamily are sorted by alphabetical order.

Species/Locality	Amezketa	Tolosa	Aia	Berrobi	Zalla	Legutiano
Coleoptera: Curculionidae: Scolytinae						
Dryocoetes autographus (Ratzeburg)	5	0	0	1	0	0
Dryocoetes villosus (F.)	9	0	3	2	0	0
Gnathotrichus materiarius (Fitch)	249	7	112	23	22	23
Hylastes ater (Paykull)	22	0	0	3	0	22
Hylurgops palliatus (Gyllenhal)	2	0	1	0	0	0
Hylurgus ligniperda (F.)	137	20	33	3	1	3
Kissophagus hederae (Schmitt)	3	0	0	0	0	0
Orthotomicus erosus (Wollaston)	111	14	57	2	26	95
Orthotomicus laricis (F.)	86	0	18	7	12	1
Pityogenes calcaratus (Eichhoff)	11	1	0	0	9	54
Polygraphus poligraphus (L.)*	0	0	0	1	0	0
Xyleborinus saxeseni (Ratzeburg)	0	0	0	0	0	16
Xyleborus eurygraphus (Ratzeburg)	35	0	11	0	0	0
Xyleborus dryographus (Ratzeburg)	1	0	7	1	0	0
Xylosandrus germanus (Blandford)	1	0	4	2	0	0
Coleoptera: Cerambycidae						
Monochamus galloprovincialis (Olivier)	12	0	2	0	0	0
Monochamus sutor (L.)	28	0	4	0	0	1
Coleoptera: Cleridae						
Allonyx quadrimaculatus (Schaller)	2	0	0	0	0	0
Clerus mutillarius F.	1	0	0	0	0	0
Thanasimus formicarius (L.)	337	157	33	43	25	45

*Indicates first record for the Iberian Peninsula.

disease in different countries, including in Europe (Portugal and Spain) [23–26]. The kairomonal attraction to bark beetle pheromone components has been previously reported in some long-horned beetles, including *M. galloprovincialis* in Spain, another North American species of the genus [27–29].

The checkered beetle *Thanasimus formicarius* (L.) (Coleoptera: Cleridae) was the most common predator found in traps (527 individuals in *P. pinaster* sawmills and 113 in *P. radiata* sawmills). This insect is a common predator of European conifer bark beetles [30], and it is capable of locating their preys by detecting bark beetle produced-pheromones as kairomonal signals [31]. Moreover, it has been reported that they recognize conifer volatiles and even volatiles from angiosperm trees that act as nonhost volatiles to conifer bark beetles [32]. *Allonyx quadrimaculatus* (Schaller) is also considered as a predatorof *Tomicus*

piniperda L. [33], although there are not concrete studies about the mechanisms involved in prey detection.

As in other species of the genus, management programs should be focused on minimizing attacks on living trees, the sanitation of infested trees and the establishment of a trapping system [7]. The use of semiochemicals with antiaggregative effects should be considered as a useful management tool for trees protection. (1*S*, 4*S*)-(-)-Verbenone (4,6,6-trimethylbicyclo-[3.1.1]hept-3-en-2-one, hereafter (-)-verbenone), has been demonstrated to be capable of disrupting the pheromone-mediated attraction of *I. sexdentatus* [16, 34]. Romón et al. [16] detected a significant negative dose-dependent relationship between different (-)-verbenone release rates (0.01, 0.2, 1.8, and 3.1 mg/24 h) and catches of *I. sexdentatus* in a *P. radiata* stand. Etxebeste and Pajares [34] also found significant reduction in catches when testing (-)-verbenone at 2 and 40 mg/day in a mixed pine stand (ca. 40-year-old *P. pinaster* with younger ca. 30-year-old *P. sylvestris*). In addition, the spyroketal *trans*-7-methyl-1,6-dioxaspiro[4.5]decane (commonly known as *trans*-conophthorin) has also shown promising results. There are evidences of its electrophysiological detection by *I. sexdentatus* [35],and the antiaggregative effect is supported by field assays, although with some disparities. Despite Jactel et al. did not find any significant reduction in trap catches when testing *trans*-conophthorin at 5 mg/day [35], a 16-time lower release rate (i.e., 0.3 mg/day) is capable of reducing the response of *I. sexdentatus* to aggregation pheromone [34]. Moreover, *trans*-conophthorin seems to achieve stronger effects when combined either with (-)-verbenone or NHV alcohols [34, 35]. Thus, taken into account these results, we suggest the development of "push-pull" strategies [36], using pheromone-baited traps inside the park (to favor insect mass trapping) and blends of disruptant semiochemicals at the edges of close pine stands, in order to repel incoming beetles. Long-time buildup of logs should also be not recommended. Future field studies are needed to evaluate the impact of these incoming *I. sexdentatus* populations upon local conifer stands.

Acknowledgments

The authors would like to express thanks to the Basque Country Forest Confederation for financial support and the owners of sampling sawmills for allowing our experimental work. They would like to thank to members of NEIKER for technical assistance. The present study was funded by The Government of the Basque Country.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 812091, 9 pages
doi:10.1155/2012/812091

Research Article

The Impact of Variation in Foliar Constituents of Sunflower on Development and Reproduction of *Diacrisia casignetum* Kollar (Lepidoptera: Arctiidae)

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Received 22 March 2012; Revised 18 May 2012; Accepted 24 May 2012

Academic Editor: G. B. Dunphy

Effects of feeding on young, mature, and senescent sunflower leaves were studied under laboratory conditions (27 ± 0.5°C, 12L : 12D, 65 ± 5% RH) to evaluate the impact of variation of nutrients on larval food utilization efficiency, larval and pupal development and survival, longevity, and fecundity of *Diacrisia casignetum* Kollar. The growth rate, which is the ratio between the dry weight gain of insect and duration of experimental period, of *D. casignetum* was in the order of mature leaf > young leaf > senescent leaf of sunflower. This was correlated with nutrient constituents of three kinds of sunflower leaves, which was measured by various biochemical analyses described elsewhere in the text. Total carbohydrates, proteins, lipids, nitrogen, amino acids, and water content are in greater amount in mature leaves when compared to young and senescent leaves, whereas phenol content was highest in young leaves than mature leaves. Hence, higher amount of total carbohydrates, proteins, lipids, nitrogen, amino acids including water and lower amount of total phenol content in mature leaves have influenced higher growth rate, less developmental time, and higher fecundity of *D. casignetum*.

1. Introduction

Diacrisia casignetum Kollar (Lepidoptera: Arctiidae) is an important economic pest in India and many other Asian countries [1]. The species is highly polyphagous, which is one of the major factors contributing to the pest status of this moth. Important agricultural crops such as sunflower, jute, sesame, and castor are among the host records in India. The occurrence of this arctiid moth has long been recorded in sunflower plant, and now it has been proved a serious defoliator in recent years [1]. The larvae of this arctiid moth feeds gregariously on sunflower leaves leaving mid ribs only and causes economic losses of this oil seed crop based on the crop stage and infestation level in the field [1].

Host plant quality is a key determinant of herbivorous insect which affects fecundity, growth rate, and development of insect [2]. Variation in development, survival, and fecundity of phytophagous insects is mainly due to variation in qualitative and quantitative amounts of nutrients among host plants including change in the nutritional quality of leaves within a particular host plant during its different developmental stages [3]. Herbivores of polyphagous nature often show better development, survival, and reproduction on mature leaves than young leaves within a plant because of higher level of toxic secondary substances in young leaves [4, 5]. Hence, it will be interesting to observe whether mature leaves of sunflower are more nutritious to this arctiid moth than young and senescent sunflower leaves. The previous study on the biological parameters of *D. casignetum* on sunflower and hempweed leaves indicated influences of host plants, that is, a crop and a weed, on development of this insect pest [6]. But there are no reports on food utilization, development, and reproduction of *D. casignetum* by feeding on young, mature, and senescent sunflower leaves. So, in this study, we examined the role of sunflower leaves throughout its developmental stage on

food utilization efficiency, larval and pupal development and survival, longevity, and fecundity of the arctiid moth, *D. casignetum*.

2. Materials and Methods

2.1. Insect Rearing. *Diacrisia casignetum* adults (male and female) were originally collected from the field near Chinsurah Rice Research Center (22°53′ *N*, 88°23′ *E*), West Bengal, India and were subsequently reared in cages (50 cm × 50 cm × 50 cm) containing fresh young (1–2 weeks old), mature (2–4 weeks old) and senescent (5–7 weeks old) sunflower (cv. PAC-36) leaves separately for oviposition of *D. casignetum*. Two pairs of newly emerged adult males and females were released in sterilized glass jars (20 cm × 10 cm) covered with a fine nylon net at 27 ± 0.5°C, 12L: 12D, 65 ± 5% relative humidity. The adults were fed with 10% sucrose solution through a cotton ball in a small Petri dish (2 cm × 1 cm). The host plant leaves (i.e., young, mature and senescent leaf) used in this study were given for oviposition separately in different sterilized glass jars. To maintain natural condition of leaves, a moist piece of cotton was placed around the cut ends of leaves followed by wrapping with aluminum foil to prevent moisture loss. Fresh leaves were given daily by replacing the previous one until eggs were laid by the test insects, and the eggs with each kind of host plant leaves were placed in new sterilized glass jars separately. *Diacrisia casignetum* larvae developed from the eggs had been fed with the respective kind of sunflower leaf separately for three generations, and from the fourth generations, the comparative rate of development of this insect on each kind of sunflower leaf was enumerated depending on the total body weight and duration of postembryonic development. To study the duration of larval development, the fourth generation eggs were separated and reared separately in sterilized glass jars containing 20 larvae on each kind of leaf and observations were noted on their incubation period and duration of each larval stage during their respective development.

The weight gain of insects, the weight of food consumed, and the weight of faeces produced were determined in a monopan balance (±0.01 mg). Fourth generation larvae of approximately same size were selected and weighed initially and were reared separately on young, mature, and senescent sunflower leaves into separate sterilized glass jars. They were allowed feeding on weighed quantity of each kind of leaf for 24 h separately as no-choice bioassay and were reweighed. The fresh weight gain during each instar was estimated by determining the differences in weight of larvae (by subtracting initial and final weight during the period of study). Ten larvae from each of the instar fed on each kind of leaf were weighed and dried in a hot air oven and weighed again to determine the percentage dry conversion value which was used to estimate dry weight of experimental larvae. The three kinds of sunflower leaves were left after 24 h of insect feeding and were oven dried, and weighed to determine dry weight gain of the diet given to the larvae. Sample leaves from the each kind of leaf were weighed, oven dried and reweighed to estimate percent dry weight conversion values to allow estimation of the dry weight of the diet supplied to the larvae. The quantity of the food consumed was estimated by determining the difference between the dry weight of diet remaining at the end of each experiment and total dry weight of diet initially provided. Twenty larvae were used in each kind of sunflower leaf treatment for each instar, and each instar had five replicates with a particular type of leaf.

2.2. Oviposition Assay. This experiment was conducted by taking laboratory reared third generation male and female adults of same age that were reared on the three kinds of sunflower leaves separately. The adults were released into separate sterilized glass jars (20 cm × 10 cm) at a sex ratio of 1 : 1 to note their mating, egg laying behavior, and further developmental stages. The adults were fed with 10% sucrose solution through a cotton ball in a small glass Petri dish (2 cm × 1 cm). After mating, the females were allowed to oviposit for 48 h, and the number of eggs was recorded for each kind of leaf per female.

2.3. Food Utilization Indices. Food utilization indices (all based on dry weight) were calculated based on the formulas of Waldbauer [7] with slight modifications [8–11] to assess the feeding efficiencies of *D. casignetum* as follows:

$$\text{growth rate (GR)} = P/Q,$$

$$\text{consumption rate (CR)} = R/Q,$$

$$\text{relative growth rate (RGR)} = P/QS,$$

$$\text{consumption index (CI)} = R/QS,$$

$$\text{approximate digestibility (AD) (\%)} = 100\,(R-T)/R,$$

$$\text{efficiency of conversion of ingested food (ECI) (\%)} = 100\,P/R,$$

$$\text{efficiency of conversion of digested food (ECD) (\%)} = 100\,P/(R-T),$$

$$\text{hatchability (\%)} = 100\,A/B,$$

$$\text{larval survivability (LS) (\%)} = 100\,N_b/N_a,$$

$$\text{effective rate of rearing (ERR) (\%)} = 100\,C/D,$$

$$\text{moth emergence (ME) (\%)} = 100\,E/C,$$

$$\text{feeding index (FI)} = F/G,$$

where P: dry weight gain of insect; Q: duration of experimental period; R: dry weight of food eaten; S: mean dry weight of insect during time Q; T: dry weight of faeces produced; A: number of eggs hatched; B: number of eggs laid by per female; N_a: number of larvae in beginning of instar; N_b: number of larvae in succeeding instar; C: number of cocoons harvested; D: number of larvae brushed (number of last instar larvae reached pupation); E: number of moths emerged; F: pupal weight; G: total weight of food consumed by the larvae.

Table 1: Life cycle of *D. casignetum* reared on young, mature, and senescent sunflower leaves.

Life cycle	Young	Mature	Senescent	$F_{2,12}$	P
Larval duration (days)					
I	3.16 ± 0.04^a	3.06 ± 0.15^a	3.36 ± 0.05^b	12.131	0.01
II	3.08 ± 0.04^a	3.06 ± 0.01^a	3.28 ± 0.07^b	5.331	0.05
III	3.94 ± 0.09	3.83 ± 0.01	4.05 ± 0.02	3.281	n.s.
IV	3.14 ± 0.07	3.00 ± 0.01^a	3.28 ± 0.07^b	5.297	0.05
V	3.01 ± 0.03	3.00 ± 0.01^a	3.16 ± 0.04^b	6.468	0.05
VI	4.91 ± 0.04	4.847 ± 0.01^a	5.08 ± 0.04^b	8.928	0.01
Total larval duration	21.25 ± 0.11^a	20.81 ± 0.02^b	22.22 ± 0.07^c	81.552	0.0001
Prepupal duration	1.83 ± 0.04	1.76 ± 0.01	1.97 ± 0.02	3.163	n.s.
Pupal duration	9.13 ± 0.05^a	9.00 ± 0.01^a	9.55 ± 0.02^b	46.380	0.0001
Male longevity	4.00 ± 0.04^a	4.01 ± 0.01^a	3.02 ± 0.02^b	318.056	0.0001
Female longevity	4.41 ± 0.17^a	4.66 ± 0.09^a	4.00 ± 0.04^b	10.243	0.01

Mean ± SE of five observations. Within the rows, means followed by different letters are significantly different.

2.4. Biochemical Analysis of Leaves. The sunflower leaves were obtained from a 2.5 × 2.5 m plot of the sunflower field near Chinsurah Rice Research Centre (22°53′ *N*, 88°23′ *E*), West Bengal, India that was free from insecticide or herbicide, but weeds were removed by hand-picking method. The variability of nutritional quality of three kinds of sunflower leaves (i.e., young, mature, and senescent) was estimated by subjecting the leaves to various biochemical analysis described elsewhere in the text, such as total carbohydrates [12], total proteins [13], total lipids [14], total amino acids [15], total nitrogen [16], and total phenol [17]. The determination of each biochemical analysis was repeated for five times.

2.5. Estimation of Moisture Content. One gram of each kind of leaf was placed separately in a hot-air oven at 50 ± 1°C temperature for 72 h, and materials that showed constant dry weight were removed from the oven and weighed in a monopan balance (±0.01 mg). Differences in the fresh and dry weights were used to determine the percent water content of each kind of leaf. The moisture content was repeated for five times for each host leave.

2.6. Statistical Analysis. All the data on life history parameters of *D. casignetum* and biochemical analysis of three host leaves were analyzed using one-way analysis of variance (ANOVA). Means associated with all the data for each variable were separated using the Tukey test when significant values were obtained [18].

3. Results

3.1. Life Cycle of Diacrisia casignetum Reared on Young, Mature, and Senescent Sunflower Leaves. Data on life history parameters of *D. casignetum* reared on young, mature, and senescent leaves of *H. annuus* is presented in Table 1. The total larval developmental duration of *D. casignetum* was significantly affected by feeding on young, mature, and senescent sunflower leaves and was longest on senescent leaves and shortest on mature leaves ($F_{2,12} = 81.552$, $P < 0.0001$). Among the six instars, the developmental time of third instars did not vary significantly by feeding on young, mature, and senescent leaves ($F_{2,12} = 3.281$, $P > 0.05$), whereas the sixth instar took longer developmental time compared to other instars when reared with three kinds of sunflower leaves. Prepupal duration was not significantly different ($F_{2,12} = 3.163$, $P > 0.05$) when larvae were fed with three types of leaves, but pupal duration was significantly reduced in young and mature leaves than on senescent leaves ($F_{2,12} = 46.380$, $P < 0.0001$). The longevities of both male and female were significantly affected by three kinds of leaves on which their larva fed (male: $F_{2,12} = 318.056$, $P < 0.0001$; female: $F_{2,12} = 10.243$, $P < 0.01$) (Table 1). Both the male and female longevities were better in young and mature leaves than senescent leaves. Fecundity was highest in insects which were reared with mature leaves (number of eggs, 670 ± 20.13) and less on senescent leaves (number of eggs, 523 ± 33.65) ($F_{2,12} = 8.644$, $P < 0.01$) (Figure 1).

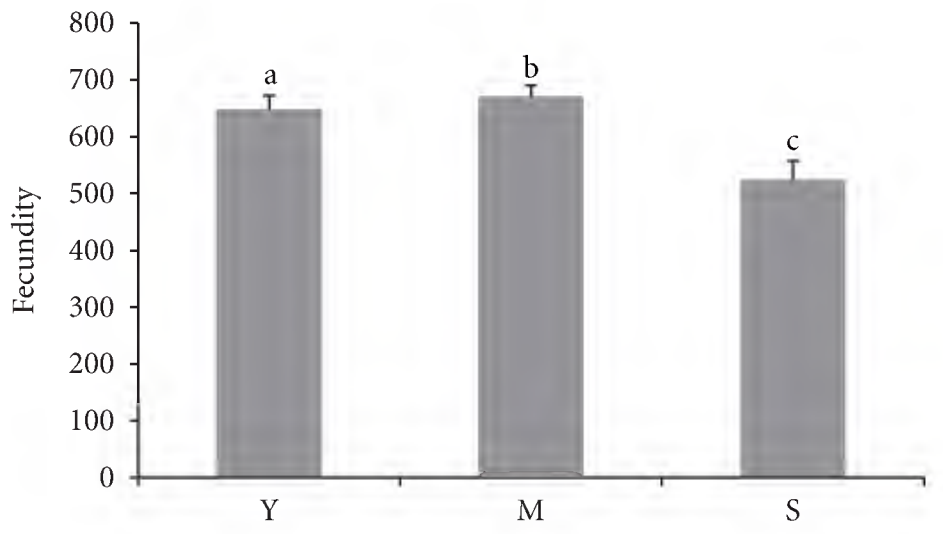

Figure 1: Total eggs per female *D. casignetum* on young (Y), mature (M), and senescent (S) sunflower leaves. Means ± SE of five observations. The different letters over the three bars indicate that the means are significantly different at $P < 0.05$.

Table 2: Food utilization efficiency measures of first instar larva of *D. casignetum* reared on young, mature and senescent sunflower leaves.

Parameter	Young	Mature	Senescent	$F_{2,12}$	P
GR (mg/day)	0.06 ± 0.00^a	0.08 ± 0.02^b	0.04 ± 0.00^c	9.859	0.01
CR (mg/day)	15.17 ± 0.05^a	17.02 ± 0.12^b	13.26 ± 0.03^c	551.283	0.0001
RGR (mg/day)	0.38 ± 0.02^a	0.32 ± 0.01^b	0.31 ± 0.03^b	8.234	0.01
CI (mg/day)	84.46 ± 2.58^a	69.60 ± 1.48^b	83.13 ± 3.07^a	11.063	0.01
AD (%)	89.56 ± 0.15	89.61 ± 0.40	89.09 ± 0.19	1.048	n.s.
ECI (%)	0.46 ± 0.03^a	0.47 ± 0.02^a	0.37 ± 0.04^b	5.931	0.05
ECD (%)	0.51 ± 0.04^a	0.52 ± 0.03^a	0.42 ± 0.04^b	5.892	0.05
LS (%)	98.26 ± 0.97^a	98.29 ± 0.93^a	92.66 ± 1.89^b	5.834	0.05

Mean ± SE of five observations. Within the rows means followed by different letters are significantly different. Food utilization efficiency measures: GR: Growth rate, CR: Consumption rate, RGR: Relative growth rate, CI: Consumption index, AD: Approximate digestibility, ECI: Efficiency of conversion of ingested food, ECD: Efficiency of conversion of digested food, LS: Larval survivability.

Table 3: Food utilization efficiency measures of second instar larva of *D. casignetum* reared on young, mature, and senescent sunflower leaves.

Parameter	Young	Mature	Senescent	$F_{2,12}$	P
GR (mg/day)	0.43 ± 0.00^a	0.48 ± 0.02^b	0.38 ± 0.01^c	10.282	0.01
CR (mg/day)	29.74 ± 0.05	29.31 ± 0.73	28.59 ± 0.08	1.849	n.s.
RGR (mg/day)	0.43 ± 0.00^a	0.36 ± 0.10^b	0.45 ± 0.02^a	8.391	0.01
CI (mg/day)	30.04 ± 0.30^a	22.41 ± 0.53^b	29.79 ± 0.44^a	98.241	0.0001
AD (%)	84.94 ± 0.24^a	84.53 ± 0.47^a	82.88 ± 0.10^b	11.966	0.01
ECI (%)	1.44 ± 0.01^a	1.67 ± 0.01^b	1.50 ± 0.04^a	14.521	0.001
ECD (%)	1.70 ± 0.02^a	1.97 ± 0.01^b	1.81 ± 0.05^c	11.760	0.01
LS (%)	88.72 ± 1.45^a	91.62 ± 0.42^b	83.55 ± 0.79^c	9.064	0.01

Mean ± SE of five observations. Within the rows, means followed by different letters are significantly different. Food utilization efficiency measures: GR: growth rate, CR: consumption rate, RGR: relative growth rate, CI: consumption index, AD: approximate digestibility, ECI: efficiency of conversion of ingested food, ECD: efficiency of conversion of digested food, LS: larval survivability.

3.2. Food Utilization Efficiency Measures. Food utilization efficiency measures of first instar larvae of *D. casignetum* are given in Table 2. The GR was significantly higher in insects fed with mature leaves followed by young and senescent leaves ($F_{2,12} = 9.859$, $P < 0.01$). There were significant differences in CR between all the treatments ($F_{2,12} = 551.283$, $P < 0.0001$). CR was highest in mature leaves followed by young and senescent leaves, whereas RGR values were greater on young leaves than other two kinds of leaves ($F_{2,12} = 8.234$, $P < 0.01$). A higher value of CI was observed in insects when reared on young leaves, whereas mature-leaf-fed insects showed lower CI values ($F_{2,12} = 11.063$, $P < 0.01$). The AD was almost same when the insects were fed with three types of leaves ($F_{2,12} = 1.048$, $P > 0.05$). ECI ($F_{2,12} = 5.931$, $P < 0.05$) and ECD ($F_{2,12} = 5.892$, $P < 0.05$) values were higher on insects fed with young and mature leaves and lower on senescent leaves. The larval survivability was greater in insects that were fed with young and mature leaves rather than senescent leaves ($F_{2,12} = 5.834$, $P < 0.05$).

Table 3 presents food utilization measures for second instar larvae of *D. casignetum*. GR was greatest in insects fed with mature leaves and lowest in senescent leaves ($F_{2,12} = 10.282$, $P < 0.01$). The CR did not vary significantly between all the treatments ($F_{2,12} = 1.849$, $P > 0.05$). Higher values of RGR were recorded in young and senescent leaves than mature leaves ($F_{2,12} = 8.391$, $P < 0.01$). CI was greater in young and senescent leaves, whereas it was lower in mature leaves ($F_{2,12} = 98.241$, $P < 0.0001$). Insects reared on young and mature leaves showed higher values of AD, whereas the value of this index was lower in senescent leaves ($F_{2,12} = 11.966$, $P < 0.01$). ECI ($F_{2,12} = 14.521$, $P < 0.001$) and ECD ($F_{2,12} = 11.760$, $P < 0.01$) values of mature-leaf-fed insects were greater than those of insects fed on young and senescent sunflower leaves. The LS index was higher on mature leaves followed by young and senescent sunflower leaves ($F_{2,12} = 9.064$, $P < 0.01$).

The data given on Table 4 provides food utilization efficiency measures of third instar larvae of *D. casignetum* reared on three kinds of sunflower leaves. Greater value of GR was recorded for insects fed on mature leaves and significantly lower in senescent leaves ($F_{2,12} = 6.940$, $P < 0.01$). Food consumption (CR) was greatest when insects were fed with mature leaves and least when fed with senescent leaves ($F_{2,12} = 1916.802$, $P < 0.0001$). Based on the value of AD index, three kinds of leaves can be arranged in order of food quality as mature > young > senescent leaves ($F_{2,12} = 515.709$, $P < 0.0001$). The value of ECI was greater in insects fed on young and senescent leaves, whereas it was reduced in case of mature-leaf-fed insects ($F_{2,12} = 57.141$, $P < 0.0001$). ECD values were higher on senescent leaves and

Table 4: Food utilization efficiency measures of third instar larva of *D. casignetum* reared on young, mature, and senescent sunflower leaves.

Parameter	Young	Mature	Senescent	$F_{2,12}$	P
GR (mg/day)	0.85 ± 0.00^{a}	0.90 ± 0.01^{b}	0.78 ± 0.00^{c}	6.940	0.01
CR (mg/day)	74.81 ± 0.02^{a}	91.66 ± 0.41^{b}	72.16 ± 0.02^{c}	1916.802	0.0001
RGR (mg/day)	0.26 ± 0.00^{a}	0.24 ± 0.02^{b}	0.27 ± 0.00^{c}	139.082	0.0001
CI (mg/day)	21.52 ± 0.12^{a}	22.66 ± 0.54^{ab}	23.16 ± 0.14^{b}	6.282	0.05
AD (%)	56.59 ± 0.02^{a}	65.52 ± 0.61^{b}	49.39 ± 0.05^{c}	515.709	0.0001
ECI (%)	1.20 ± 0.01^{a}	0.96 ± 0.02^{b}	1.18 ± 0.01^{a}	57.141	0.0001
ECD (%)	2.13 ± 0.02^{a}	1.56 ± 0.02^{b}	2.40 ± 0.02^{c}	354.519	0.0001
LS (%)	87.60 ± 2.40	86.27 ± 0.49	84.85 ± 2.84	0.571	n.s.

Mean ± SE of five observations. Within the rows, means followed by different letters are significantly different. Food utilization efficiency measures: GR: growth rate, CR: consumption rate, RGR: relative growth rate, CI: consumption index, AD: approximate digestibility, ECI: efficiency of conversion of ingested food, ECD: efficiency of conversion of digested food, LS: larval survivability.

Table 5: Food utilization efficiency measures of fourth instar larva of *D. casignetum* reared on young, mature, and senescent sunflower leaves.

Parameter	Young	Mature	Senescent	$F_{2,12}$	P
GR (mg/day)	6.13 ± 0.01^{a}	6.69 ± 0.29^{b}	5.69 ± 0.01^{c}	6.069	0.05
CR (mg/day)	164.29 ± 0.02^{a}	175.50 ± 1.13^{b}	159.29 ± 0.02^{c}	160.278	0.0001
RGR (mg/day)	0.40 ± 0.00^{a}	0.34 ± 0.03^{b}	0.41 ± 0.00^{a}	158.179	0.0001
CI (mg/day)	10.84 ± 0.01^{a}	8.97 ± 0.08^{b}	11.23 ± 0.00^{c}	549.641	0.0001
AD (%)	70.09 ± 0.02^{a}	71.29 ± 0.58^{a}	67.34 ± 0.00^{b}	35.232	0.0001
ECI (%)	3.73 ± 0.00	3.82 ± 0.46	3.70 ± 0.00	0.053	n.s.
ECD (%)	5.32 ± 0.01	5.36 ± 0.16	5.49 ± 0.01	0.861	n.s.
LS (%)	92.74 ± 2.24^{a}	92.95 ± 0.08^{a}	85.85 ± 2.48^{b}	4.356	0.05

Mean ± SE of five observations. Within the rows, means followed by different letters are significantly different. Food utilization efficiency measures: GR: growth rate, CR: consumption rate, RGR: relative growth rate, CI: consumption index, AD: approximate digestibility, ECI: efficiency of conversion of ingested food, ECD: efficiency of conversion of digested food, LS: larval survivability.

lower on mature leaves ($F_{2,12} = 354.519$, $P < 0.0001$). The LS value did not vary significantly when the larvae were reared on three kinds of leaves ($F_{2,12} = 0.571$, $P > 0.05$).

Table 5 provides food utilization efficiency measures for fourth instar larvae of *D. casignetum* fed on young, mature, and senescent leaves. A higher GR was found for insects that were fed on mature leaves ($F_{2,12} = 6.069$, $P < 0.05$). Insects fed with mature leaves showed a higher value of food consumption (CR), whereas insects reared with senescent leaves showed a lower value of this index ($F_{2,12} = 160.278$, $P < 0.0001$). Higher RGR values were recorded on young and senescent leaves than mature leaves ($F_{2,12} = 158.179$, $P < 0.0001$). CI values were higher for insects given senescent leaves ($F_{2,12} = 549.641$, $P < 0.0001$). A higher value of AD was found in young and mature leaves, while AD was lower when the insects were fed with senescent leaves ($F_{2,12} = 35.232$, $P < 0.0001$). ECI ($F_{2,12} = 0.053$, $P > 0.05$) and ECD ($F_{2,12} = 0.861$, $P > 0.05$) values did not vary significantly when the larvae were reared with three kinds of leaves. LS was higher when the insects were fed with young and mature leaves, while a lower LS was recorded when fed with senescent leaves ($F_{2,12} = 4.356$, $P < 0.05$).

Food utilization measures for fifth instar larvae of *D. casignetum* are given in Table 6. Insects reared on mature leaves showed higher values of GR followed by young and senescent sunflower leaves ($F_{2,12} = 616.978$, $P < 0.0001$). A higher value of CI was observed in young leaves, whereas mature-leaf-fed insects showed lower CI values ($F_{2,12} = 526.015$, $P < 0.0001$). Higher values of AD were observed on young and mature leaves ($F_{2,12} = 69.189$, $P < 0.0001$). Higher ECD ($F_{2,12} = 48.374$, $P < 0.0001$) and ECI ($F_{2,12} = 65.861$, $P < 0.0001$) values were evident on young and mature leaf than senescent leaves. LS values of young- and mature-leaf-fed insects were higher than those of insects fed on senescent leaf ($F_{2,12} = 5.234$, $P < 0.05$).

Table 7 provides food utilization measures for sixth instar larvae of *D. casignetum*. A higher GR was found for insects, were fed on mature leaves ($F_{2,12} = 28.443$, $P < 0.0001$). Insects fed with mature leaf showed a higher rate of food consumption (CR) followed by young and senescent leaf ($F_{2,12} = 311.005$, $P < 0.0001$). CI was greater in senescent leaf than the other two kinds of leaves ($F_{2,12} = 15.203$, $P < 0.001$). Higher values of AD were observed in young and mature leaf, while it was lower when the insects were fed with senescent leaf ($F_{2,12} = 56.450$, $P < 0.0001$). The value of ECI was greater in insects that fed on young leaves ($F_{2,12} = 59.537$, $P < 0.0001$), whereas it was reduced in case of mature-leaf-fed insects. ECD values were higher on young and senescent leaves than on mature leaf ($F_{2,12} = 25.973$, $P < 0.0001$). LS values were higher in insects that were reared on young and mature leaves, while it was lower on senescent leaf-fed insects ($F_{2,12} = 5.121$, $P < 0.05$).

Table 6: Food utilization efficiency measures of fifth instar larva of *D. casignetum* reared on young, mature, and senescent sunflower leaves.

Parameter	Young	Mature	Senescent	$F_{2,12}$	P
GR (mg/day)	26.14 ± 0.01^a	28.15 ± 0.24^b	21.36 ± 0.01^c	616.978	0.0001
CR (mg/day)	216.44 ± 0.03^a	226.40 ± 1.51^b	202.46 ± 0.04^c	189.695	0.0001
RGR (mg/day)	0.41 ± 0.00^a	0.35 ± 0.01^b	0.34 ± 0.00^b	41.214	0.0001
CI (mg/day)	3.37 ± 0.00^a	2.79 ± 0.02^b	3.26 ± 0.00^c	526.015	0.0001
AD (%)	67.09 ± 0.01^a	67.60 ± 0.06^a	65.49 ± 0.00^b	69.189	0.0001
ECI (%)	12.07 ± 0.00^a	12.44 ± 0.21^a	10.55 ± 0.00^b	65.861	0.0001
ECD (%)	18.00 ± 0.00^a	18.62 ± 0.32^a	16.11 ± 0.00^b	48.374	0.0001
LS (%)	89.81 ± 2.02^a	90.02 ± 0.21^a	82.64 ± 3.25^b	5.234	0.05

Mean ± SE of five observations. Within the rows, means followed by different letters are significantly different. Food utilization efficiency measures: GR: growth rate, CR: consumption rate, RGR: relative growth rate, CI: consumption index, AD: approximate digestibility, ECI: efficiency of conversion of ingested food, ECD: efficiency of conversion of digested food, LS: larval survivability.

Table 7: Food utilization efficiency measures of sixth instar larva of *D. casignetum* reared on young, mature, and senescent sunflower leaves.

Parameter	Young	Mature	Senescent	$F_{2,12}$	P
GR (mg/day)	20.20 ± 0.015^a	21.41 ± 0.27^b	19.82 ± 0.01^c	28.443	0.0001
CR (mg/day)	158.86 ± 0.02^a	166.10 ± 0.63^b	153.15 ± 0.02^c	311.005	0.0001
RGR (mg/day)	0.13 ± 0.00	0.12 ± 0.01^a	0.14 ± 0.00^b	6.846	0.05
CI (mg/day)	0.97 ± 0.00^a	0.98 ± 0.02^a	1.08 ± 0.00^b	15.203	0.001
AD (%)	45.81 ± 0.02^a	45.12 ± 0.21^a	42.39 ± 0.01^b	56.450	0.0001
ECI (%)	13.47 ± 0.00^a	12.25 ± 0.13^b	12.94 ± 0.00^c	59.537	0.0001
ECD (%)	29.44 ± 0.02^a	27.71 ± 0.48^b	30.53 ± 0.02^a	25.973	0.0001
LS (%)	86.97 ± 2.80^a	86.58 ± 0.38^a	79.57 ± 1.97^b	5.121	0.05

Mean ± SE of five observations. Within the rows, means followed by different letters are significantly different. Food utilization efficiency measures: GR: growth rate, CR: consumption rate, RGR: relative growth rate, CI: consumption index, AD: approximate digestibility, ECI: efficiency of conversion of ingested food, ECD: efficiency of conversion of digested food, LS: larval survivability.

The present study revealed that the mature-leaf-fed caterpillars demonstrated greater growth rate (GR) throughout all the instars. Except for second instar, where consumption rate (CR) did not vary significantly among the three kinds of leaves, CR was higher in all the instars of mature-leaf-fed caterpillars. Relative growth rate (RGR) values displayed different patterns throughout all instars, when the insects were offered three kinds of leaves. The approximate digestibility (AD) was greater in young- and mature-leaf-fed insects during the second and last three instars, whereas AD was higher in mature-leaf-fed third instar caterpillars. Both the ECI and ECD values for the first instar were higher for young and mature leaves, whereas second instar indicated greater ECI and ECD values on mature leaf. ECI values were higher for third instar on young and mature leaves than senescent leaf, while ECD value was higher on senescent-leaf-fed insects. ECI and ECD values did not differ significantly, when fourth instar caterpillars were reared on three kinds of leaves. Fifth instar larvae showed greater ECI and ECD on young and mature leaves. ECI value for the sixth instar larvae was higher when they were given young leaves, whereas higher ECD values were recorded on young and senescent leaves. Larval survivability (LS) was higher in young and mature leaves for all the instars except for second and third instars. LS did not differ significantly in third instar larvae among three kinds of sunflower leaves, whereas second instar larvae indicated better survivability on mature leaves.

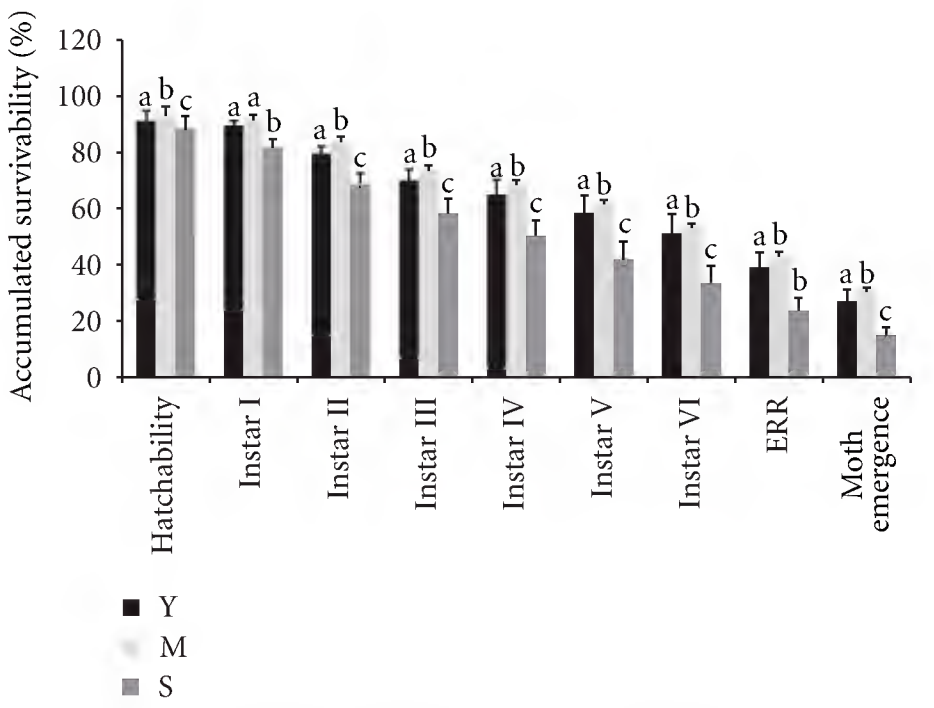

Figure 2: Accumulated survivals of *D. casignetum* on young (Y), mature (M) and senescent (S) sunflower leaves. Means ± SE of five observations. The different letters over the bars indicate that the means are significantly different at $P < 0.05$.

The hatchability percent from *D. casignetum* eggs ($F_{2,12} = 6.468$, $P < 0.05$) was highest in mature leaves and lowest in senescent leaves (Figure 2). The overall accumulated survival rate in all the larval stages was greatest when the insects were fed with mature leaves followed by young leaves and

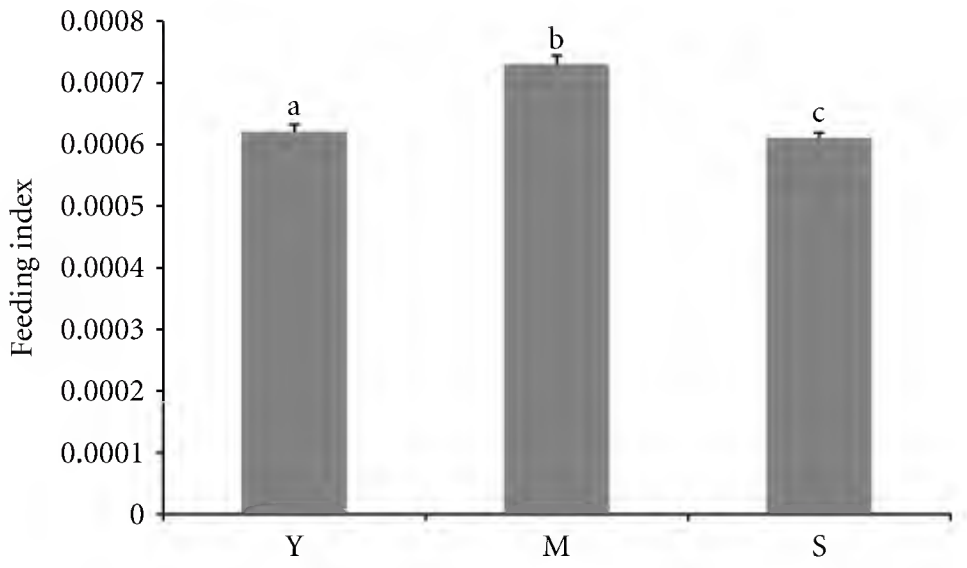

FIGURE 3: Feeding index of *D. casignetum* when reared with young (Y), mature (M), and senescent (S) sunflower leaves. Means ± SE of five observations. The different letters over the three bars indicate that the means are significantly different at $P < 0.05$.

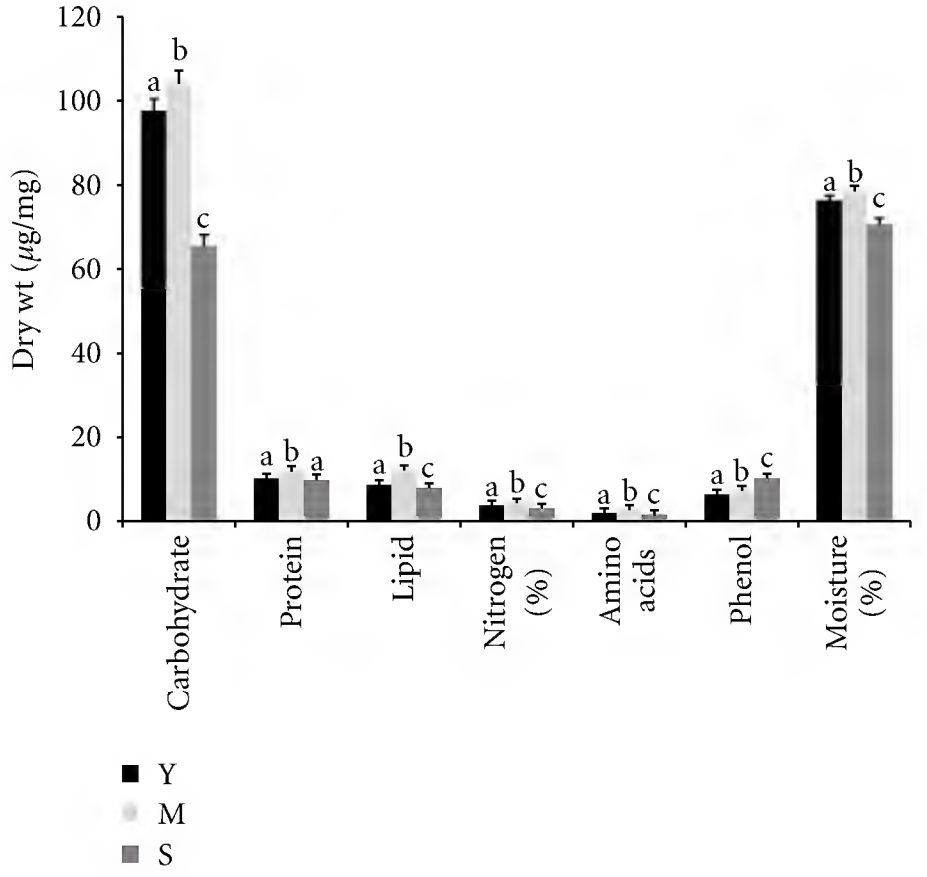

FIGURE 4: Biochemical compositions of young (Y), mature (M), and senescent (S) sunflower leaves. Means ± SE of five observations. The different letters over the three bars indicate that the means are significantly different at $P < 0.05$.

senescent leaves ($F_{2,87} = 5.545$, $P < 0.05$) (Figure 2). The ERR was comparatively higher in mature leaves followed by young and senescent leaves ($F_{2,12} = 6.176$, $P < 0.05$) (Figure 2). The emergence of adult moths from the hatched eggs was greatest when the larvae were reared on mature leaves followed by young leaves and senescent leaves ($F_{2,87} = 7.131$, $P < 0.01$). The feeding index value of mature-leaf-fed insects was higher than those of insects fed on young and senescent sunflower leaves ($F_{2,12} = 30.692$, $P < 0.0001$) (Figure 3).

3.3. Biochemical Components in Three Kinds of Leaves. Figure 4 provides variation in biochemical compositions of the three kinds of leaves. Total carbohydrate ($F_{2,12} = 544.244$, $P < 0.0001$) and protein ($F_{2,12} = 17.120$, $P < 0.001$) content varied significantly between the leaf stages and can be placed in the order of mature leaf > young leaf > senescent leaf. Lipid content was greatest in mature leaf and least in senescent leaf ($F_{2,12} = 343.125$, $P < 0.0001$). Total nitrogen ($F_{2,12} = 73.921$, $P < 0.0001$) and amino acid ($F_{2,12} = 56.611$, $P < 0.0001$) were highest in mature leaf and lowest in senescent leaf. Total phenol content was greatest in young leaf followed by mature leaf and least in senescent leaf ($F_{2,12} = 480.903$, $P < 0.0001$). Water content varied significantly between the leaves ($F_{2,12} = 146.008$, $P < 0.0001$). Mature leaf of sunflower plant had highest water content followed by young and senescent leaf.

4. Discussion

The concentration and proportion of nutrients vary considerably within a particular species throughout its different developmental stages, which influences food utilization, development, and reproduction of herbivorous insects [3, 5]. Herbivorous insects are adapted to the nutrient composition of host leaves within a particular species, so it would be expected that development and reproduction will be better on leaves which are rich in nutrients. The biochemical components, that is, carbohydrates, proteins, lipids, amino acids, nitrogen, and phenols play an important role for host selection of phytophagous insects [5, 19–22]. Host plant utilization is also influenced by the ability of insect to ingest, assimilate, and convert food into body tissues [23]. In the present study, it was observed that *D. casignetum* showed significant differences in growth rate, consumption rate, utilization efficiency, developmental time, and fecundity when they were reared on young, mature, and senescent sunflower leaves, separately.

Carbohydrate deficiency results in reduction of general vitality, activity, and growth rate of phytophagous insects even though proteins and lipids serve as an alternative source of energy [5, 24, 25]. Higher level of carbohydrate content was observed in mature sunflower leaves, and insects fed on this kind of leaf exhibited a higher growth rate. During diapause, lipids serve as primary source of energy [26]. Furthermore, lipid is an essential component of insect diet, which acts as precursors of ecdysteroid moulting hormone and provides structural role in cellular membranes and transport of lipoproteins [27]. More growth rate was observed in *D. casignetum* fed with mature leaves of sunflower plant where lipid content was also higher than young and senescent leaves.

The protein content of host leaves is generally a limiting factor for the optimal growth of phytophagous insects [5, 20]. Insects feeding on nitrogen-rich leaves had a higher growth rate than those which consume leaves containing less nitrogen [20, 22]. Mature leaves are rich in protein and nitrogen when compared to young and senescent leaves, suggesting that insects feeding on mature sunflower leaves will develop more quickly. Further, growth and reproduction of insects could be explained in part in relation to amino acid composition of diet [5, 25]. Higher level of amino acids in mature leaves would probably explain better growth and reproduction of *D. casignetum*.

Water content in host leaves plays an important role in growth rate of plant-fed caterpillars [21, 22, 28]. The mature leaves of sunflower plant had higher water content than young and senescent leaves which might have influenced the higher growth rate of *D. casignetum* feeding on them. Decreased water content in senescent leaves reduced the growth rate of *D. casignetum* than when fed with young leaves.

Phenols induce resistance in hosts against herbivory. Consumption of greater amount of phenols was found to significantly reduce adult longevity, fecundity, and retardation of larval growth [5, 25]. In this study, phenol concentration was higher in young leaves than mature leaves, suggesting that insects feeding on mature leaves will develop more quickly and will be more successful which supports the hypothesis that polyphagous species prefer mature leaves [5]. However, despite high level of phenols in mature leaves than senescent leaves, growth rate and development of *D. casignetum* were fastest when fed on mature leaves. This could possibly be attributed to the proportionately high level of readily utilizable primary substances, that is, carbohydrates, proteins, and lipids in mature leaves than senescent leaves [5].

In the present study, all nutritional indices varied when *D. casignetum* fed on three kinds of sunflower leaves. The growth rate (GR) of insects depends on efficiency of conversion of digested food; whereas a reduction in ECD indicates higher metabolic maintenance cost [11, 20, 23]. The current data reveal that all the larval instars of *D. casignetum* had higher GR on mature leaves, which would explain shorter developmental time of mature-leaf-fed caterpillars. The consumption rate (CR) of all the instars was lower when feeding on senescent leaves compared with those of mature and young leaves. The approximate digestibility (AD) is affected by water content of host leaves and decreased water content in senescent leaves caused reduction in the efficiency of nutrient digestion or absorption because the lower water level interfered with feeding and nutritional process. Though, third, fourth, and sixth instar caterpillars were efficiently converting senescent leaf tissues into their biomass as shown by larvae fed on senescent sunflower leaf tissues having lowest approximate digestibility and the maximum efficiency of conversion of digested food. This might be due to homeostatic adjustment of consumption rates and efficiency parameters of the insect which can approach ideal growth rate even though with foods of different quality [29].

In the present study, three kinds of sunflower leaves influenced survival of *D. casignetum* in the larval, pupal, and adult stage. Egg survival (percentage hatch) was the highest (93.01%) in mature leaf followed by young leaf (91.06%) and senescent leaf (88.12%). High survival rates and shorter developmental time indicate better nutritional quality of their larval food in relation to greater amount of carbohydrates, proteins, lipids, and amino acids [20]. These results of this study would probably indicate the greater survival and shorter developmental time of *D. casignetum* when they were reared on mature leaves followed by young and senescent leaves. Furthermore, the feeding index was higher in mature leaf than the other two kinds of leaves, which indicates lesser amounts of mature leaf are consumed by *D. casignetum* to produce better quality of pupal weight.

The caterpillars reared on mature leaves show more fecundity when compared to young and senescent sunflower leaves. Host plant quality is a key determinant of the fecundity of herbivorous insects. Herbivorous insects feeding on protein-rich leaves will be more successful when compared to leaves less rich in proteins. Larval dietary nitrogen and adult carbohydrate diets influence the development of male and female reproductive system of lepidopteran insects [30]. In the present study, *D. casignetum* feeding on mature leaves allocated more nutrients to egg production than when feeding on young and senescent leaves. Nutrient accumulated during larval feeding including quality and quantity of adult food influences the number of eggs laid and its quality [19]. Hence, a reduction in larval consumption may result in longer developmental time, smaller size of the adult, and ultimately the lower fecundity [20]. Therefore, we conclude that mature leaves of sunflower plant provide the best food quality of *D. casignetum*, senescent leaves the worst, and young leaves of intermediate quality.

Acknowledgments

The authors are thankful to retired Professor T. C. Banerjee of this university for identification of this insect. The financial assistance provided by University Grants Commission (F. NO. 37/615/2009), New Delhi, Government of India is gratefully acknowledged.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 261071, 7 pages
doi:10.1155/2012/261071

Research Article

Life Histories of Two Endangered Sea Skaters *Halobates matsumurai* Esaki and *Asclepios shiranui* (Esaki) (Hemiptera: Gerridae: Halobatinae)

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Received 13 March 2012; Accepted 9 May 2012

Academic Editor: Ai-Ping Liang

Sea skaters *Halobates matsumurai* Esaki and *Asclepios shiranui* (Esaki) are among the few marine insects found in Japan. For the past several decades, they have become rare in most localities and have now been designated as endangered by the government. In order to understand their adaptive strategies to the marine environment and to develop conservation measures, it is essential to know their life histories. We studied their lifecycles in Kujukushima Bay off the north coast of Kyushu (Japan) where they co-occurred in small coves along the jagged coast. They appeared to have more than one generation a year and to overwinter in the egg stage. Eggs of *H. matsumurai* were laid on natural sandstones and man-made sandstone walls along the shore, mostly above the average sea level. The eggs had very hard shells, presumably adaptive to protect them from desiccation, solar radiation, and wave action, especially during the overwintering period.

1. Introduction

Although insects are predominant on earth over other living organisms both in species numbers and in habitat diversity, they are largely unsuccessful in conquering marine environment [1–3]. *Halobates* is the only insect genus that includes species living in the open ocean [4–7]. Among 46 known species, 5 are pelagic, 39 are found in coastal areas, and 2 occur in freshwater rivers [7]. Three species of the genus *Asclepios*, a close relative of *Halobates*, are also found in coastal waters. Most coastal *Halobates* and *Asclepios* live in tropical and subtropical mangrove habitats, many of which are now lost to development or exposed to pollution. Three coastal sea skaters, *H. matsumurai* (Figure 1(a)), *H. japonicus* Esaki, and *A. shiranui* are known in Japan. They were all described by Esaki in 1924 and found in western or southwestern coasts of Japan [8–10]. During the years of postwar rapid economic growth and industrialization in Japan (~1952–1972), many habitats suitable for coastal sea skaters were destroyed, resulting in drastic decreases in number of populations and distribution ranges [11–13]. *H. matsumurai* and *A. shiranui* were rarely seen until they were rediscovered in the 1990s [11, 14, 15]. Under these circumstances, they were both designated as threatened II (VU) and threatened I (CR + EN), respectively, by the Japanese Ministry of the Environment [16].

Little is known about the lifecycles of coastal sea skaters. Previous studies were mostly based on short-term observations in the field or in the laboratory [6, 17–21]. However, in order to understand their adaptive strategies to marine environments and to develop conservation measures, it is essential to know their life histories. Our study on *Halobates matsumurai* and *Asclepios shiranui* in their natural habitat is the first carried out over an extended period. We studied their life cycles in Kujukushima Bay (Figure 1(c), Figure 2), off the north coast of Kyushu, Japan, where they often co-occur in small coves. One of the most intriguing aspects with Japanese sea skaters is that they live in the temperate climate zone,

(a)

(b)

(c)

(d)

FIGURE 1: (a) Mating pair of *H. matsumurai* (Photo by K. Yusa). (b) Egg of *H. matsumurai* laid on sandstone. Scale = 1 mm. (Photo by T. Ikawa). (c) Deep inner bay of Kujukushima area (Photo by T. Ikawa). (d) Sandstones along the shore at low tide showing exposed oviposition area for *H. matsumurai* (Photo by K. Yusa).

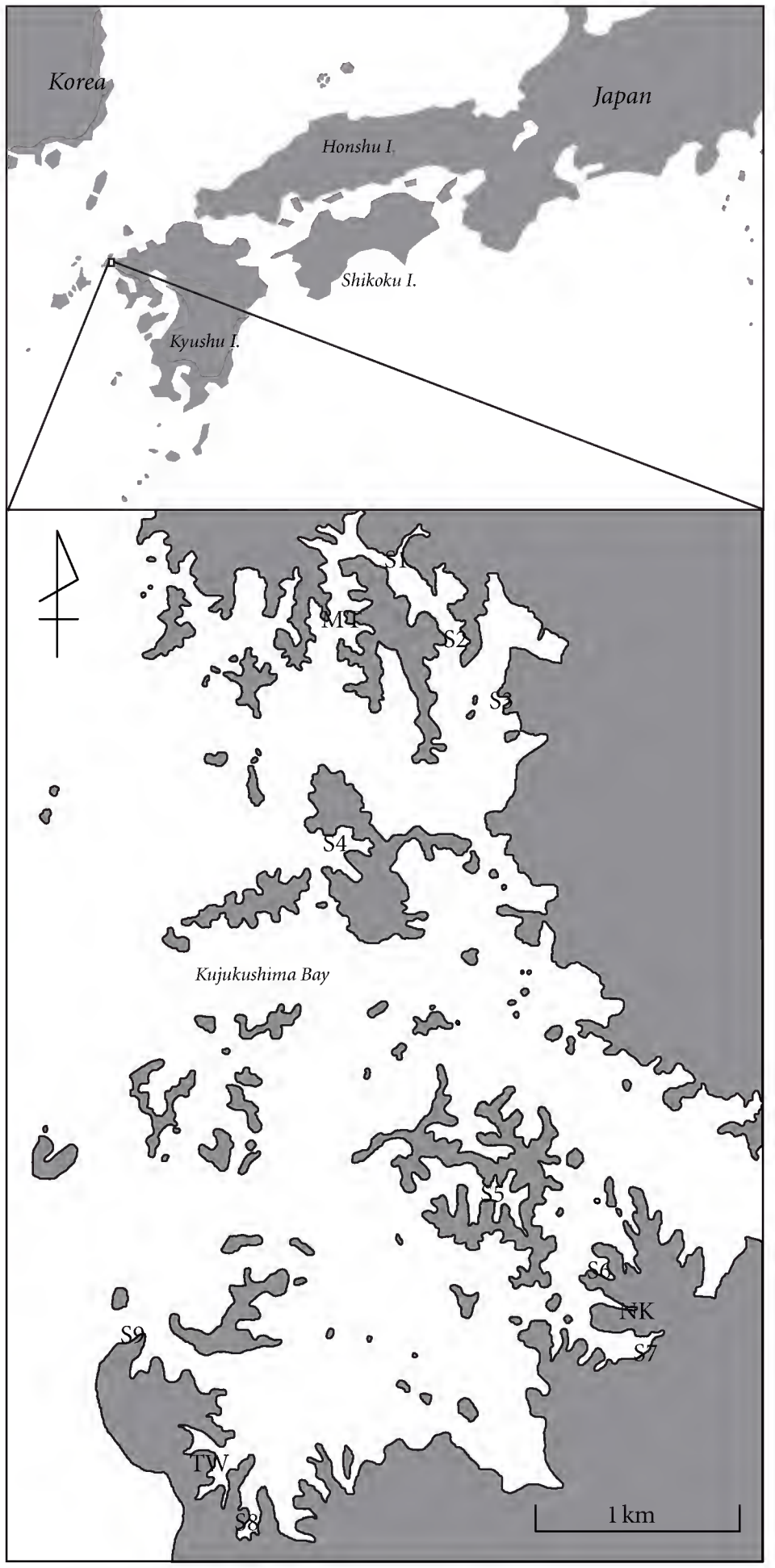

FIGURE 2: Locations of study stations MT, NK, TW and S1~S9 in Kujukushima area.

while most others are found in tropical waters. Where and in what stage they overwinter is of particular interest. Soon after this study was completed, collection of *H. matsumurai* and *A. shiranui* as well as other endangered organisms was banned. Thus, we had a unique opportunity to study their life cycles.

2. Materials and Methods

2.1. Study Site. The distribution ranges of the three Japanese sea skaters are as follows: *A. shiranui* is known in the coasts of northwestern Kyushu Is. [11]; *H. matsumurai* is found along the coasts of northwestern Kyushu Is. and western Honshu Is. [11, 22–24]; *H. japonicus* is known from the western Pacific coast of Honshu Is. to the Nansei Islands [12]. *A. shiranui* and *H. matsumurai* often co-occur in small coves or inner bays [11, 22, 24], but *H. japonicus* has never yet been found to co-occur with either *A. shiranui* or *H. matsumurai*. Only *H. matsumurai* and *A. shiranui* are known in coastal areas of Kujukushima [11, 22–24]. Kujukushima consists of more than 200 islands scattered off the northwest coast of Kyushu. The two species can be found in small coves off uninhabited jagged coastlines many of which were only

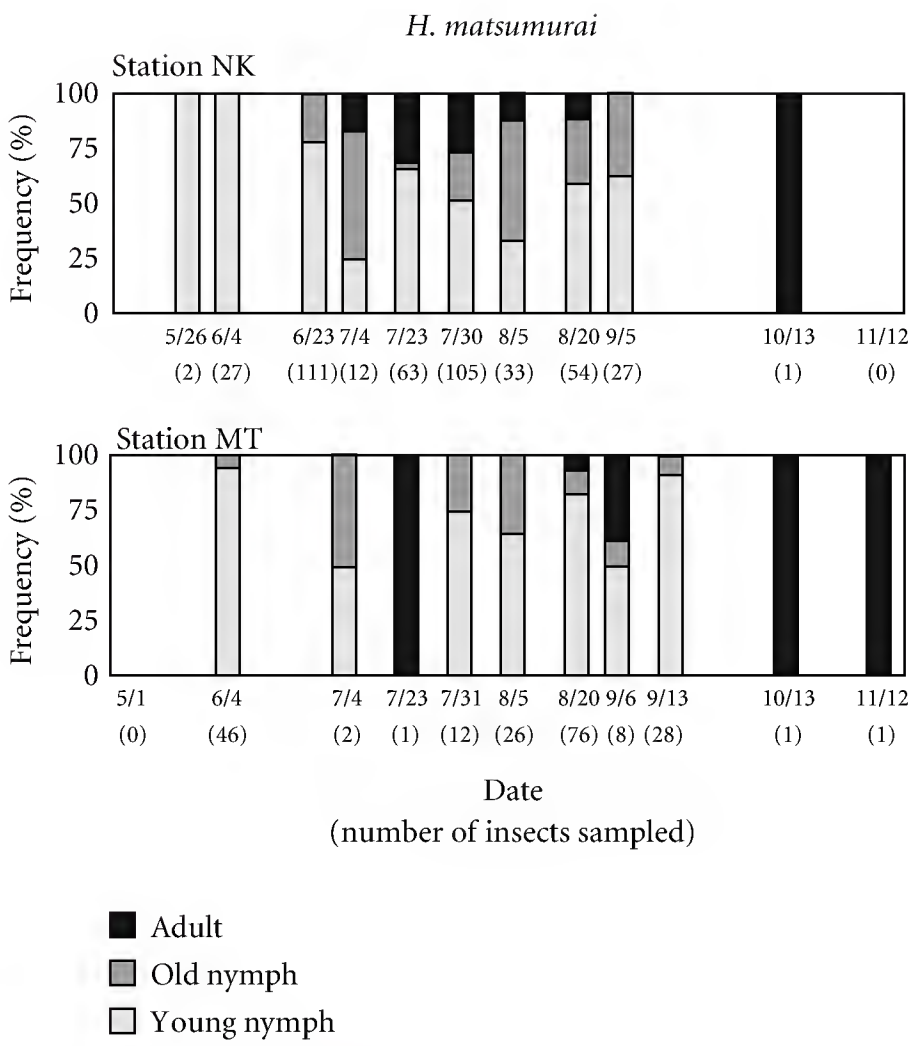

FIGURE 3: Seasonal changes in the composition of developmental stages of *H. matsumurai* at Station NK (top) and MT (bottom).

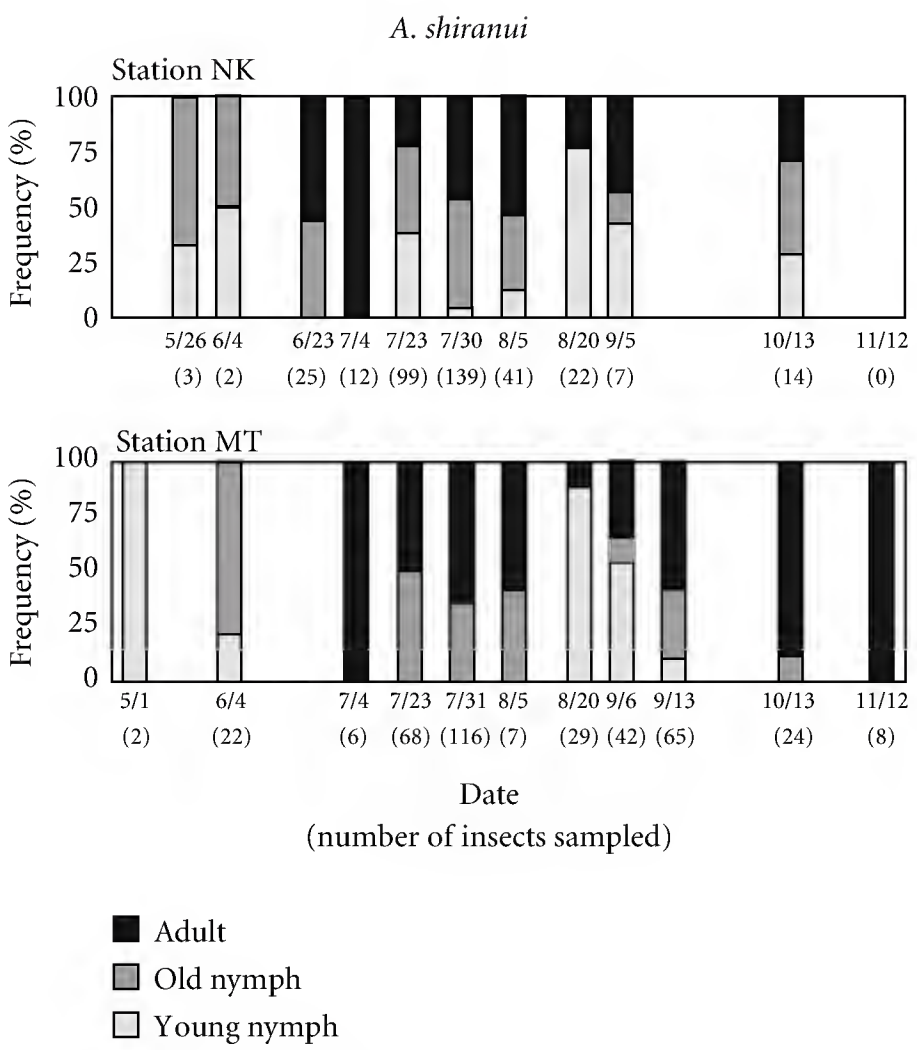

FIGURE 4: Seasonal changes in the composition of developmental stages of *A. shiranui* at Station NK (top) and MT (bottom).

accessible by boat [11, 23]. We conducted field surveys at stations MT, NK, TW, and S1~S9 in Kujukushima Bay (ca. 33°8′N 129°40′E) (Figure 2). Each station was visited by vessels owned by Kujukushima Aquarium. The studies were carried out between March 2008 and February 2009.

2.2. Oviposition Sites and Appearance of Nymphs and Adults. *H. matsumurai* females were known to lay eggs on the rocks on the shore [11, 23] but oviposition sites of *A. shiranui* were unknown. In the laboratory, females of *A. shiranui* oviposited on sandstones [Nozoe, unpublished observation]. Eggs of *A. shiranui* could be distinguished by size much smaller than those of *H. matsumurai*. Throughout our study period, we examined the presence of *H. matsumurai* eggs and also searched for *A. shiranui* eggs.

On March 4–6, 2008, we intensively searched nine sites (S1, S2, S3, S5, S6, S8, S9, TW, and NK in Figure 2) for eggs, nymphs, and adults. For eggs, we mainly searched sandstones and man-made stonewalls along the shore and some seaweeds nearby. For adults and nymphs, we looked on the sea surface and the shore. Similar surveys were carried out in late March and April at five stations: NK (March 22), S3 (March 31), S2 (April 11), S4 (April 15), and S8 (April 20).

From May to November 2008, we sampled nymphs and adults for a total of 11 times at stations NK and MT on the following dates: station NK, May 26, June 4, 23, July 4, 23, 30, Aug. 5, 20, Sept. 5, Oct. 13, Nov. 12; station MT, May 1, June 4, July 4, 23, 31, Aug. 5, 20, Sept. 6, 13, Oct. 13, Nov. 12. We collected sea skaters using insect nets with an opening of 25 cm in diameter either from a boat or from shore.

In late November, we repeated our intensive searches for eggs, nymphs, and adults at S8 and S9 (Nov. 26), and at MT, S5, and TW (Nov. 27), as we did in March and April. During the cold season (December 2008 to February 2009), in addition to stations NK and MT we searched three more sites (S4, TW, S8) in Kujukushima Bay to confirm whether nymphs or adults were present. Surveys were made once a month, that is, Dec. 13, 2008, Jan. 9, and Feb. 10, 2009.

Specimens collected were preserved in 99% ethanol for further studies in the laboratory. They were identified to species under a stereomicroscope. Adults and 5th instar nymphs were sexed, and nymphs were sorted to each of five developmental stages. The species and the developmental stages were separated based on the color pattern of dorsal side, the length of midleg femur, and the presence of genitalic organ (Ikawa et al., in preparation). Some of the adult females were dissected to count eggs in ovarioles and to measure the length and width of mature eggs.

2.3. Vertical Distributions of H. matsumurai Eggs and Duration of Submergence Underwater. The location of *H. matsumurai* eggs in relation to the sea level was studied at station TW on November 28, 2008. The eggs were laid on a man-made 1240 cm wide sandstone wall along the shore. The lower edge of the stonewall was 119 cm above the sea level and upper edge was 281 cm high. The mean sea level at Kujukushima Bay area including station TW is 164 cm [25]. Since the eggs were found on the same stonewall in March 2009, they would have remained there throughout the winter until they hatched in May or later [Ikawa et al., unpublished observation]. To estimate how long the eggs

Table 1: Average numbers of mature and submature eggs per female and length and width of mature eggs of *H. matsumurai*.

Date of sampling (2008)	N^*	No. (average ± S.D.) of eggs/female		Length and width (average ± S.D.) of mature egg	
		Mature eggs	Submature eggs	Length (mm)	Width (mm)
June 23, 30; July 4	6 (1)	4.3 ± 4.84	1.8 ± 2.48	1.59 ± 0.01	0.53 ± 0.04
Aug. 1, 20	10 (0)	7.7 ± 3.16	4.1 ± 2.02	1.61 ± 0.09	0.52 ± 0.04
Sept. 17; Oct. 14	10 (1)	5.3 ± 2.63	3.0 ± 2.49	1.73 ± 0.10	0.59 ± 0.04

* Number of females dissected. Values in parenthesis are the number of females with neither mature nor sub-mature eggs.

Table 2: Average numbers of mature and submature eggs per female and length and width of mature eggs of *A. shiranui*.

Date of sampling (2008)	N^*	No. (average ± S.D.) of eggs/female		Length and width (average ± S.D.) of mature egg	
		Mature eggs	Submature eggs	Length (mm)	Width (mm)
June 6, 23	10 (1)	1.0 ± 2.83	5.5 ± 3.66	1.16 ± 0.08	0.37 ± 0.07
July 30, 31; Aug. 1	20 (0)	7.1 ± 3.55	6.2 ± 2.43	1.20 ± 0.04	0.39 ± 0.02
Oct. 14; Nov. 20	10 (0)	5.2 ± 1.87	3.7 ± 2.06	1.33 ± 0.05	0.39 ± 0.02

* Number of females dissected. Values in parenthesis are the number of females with neither mature nor sub-mature eggs.

had been submerged underwater during the overwintering period, percentage duration of submergence of each egg from November to April was calculated using the tidal data provided by Sasebo Coast Guard Office to Kujukushima Aquarium.

3. Results

3.1. Oviposition Sites and Appearance of Nymphs and Adults. On March 4~6, 2008, we found no eggs of *A. shiranui,* while the eggs of *H. matsumurai* were found in all nine sites. *H. matsumurai* eggs were laid mainly on natural sandstones (Figure 1(b), and 1(d)) or man-made sandstone walls, and occasionally on concrete seawalls along the shore. Neither nymphs nor adults of either species were observed during these dates or in late March or April.

From May to November 2008, nymphs and adults of both species were collected at stations NK and MT. Young nymphs (1st~3rd instar nymphs) of *H. matsumurai* first appeared in May at NK and in early June at MT (Figure 3). Older nymphs (4th~5th instar nymphs) appeared in June, and adults were found from July onwards. Nymphs and adults co-occurred from July to August at NK and from late August to September at MT. In the autumn, only a small number of adults were found at both stations.

As shown in Figure 4, young nymphs of *A. shiranui* first appeared in May, and older nymphs appeared from May at NK and from June at MT. Adults were found from June at NK and from July at MT. At NK, nymphs and adults co-occurred from June to October, and no individuals were found in November. At MT, nymphs and adults co-occurred from July to October and only adults were found in November.

In late November, we found eggs of *H. matsumurai* on the sandstones and stonewalls in all five sites surveyed (S5, S8, S9, MT, TW in Figure 2). Neither nymphs nor adults of either species were observed.

From December 2008 to February 2009, we found no nymphs nor adults of either species at stations NK and MT or the other three sites (TW, S4, S8). Eggs of *H. matsumurai* were seen on the sandstones and stonewalls. Throughout the study period, we found no eggs of *A. shiranui* in the field.

3.2. Egg Load of Adult Females. Most females of *H. matsumurai* and *A. shiranui* carried mature and/or submature eggs from early summer to autumn (Tables 1 and 2). The average numbers of mature or submature eggs/female of both species were at most 7 or 8. The eggs of *H. matsumurai* were much larger than those of *A. shiranui*. Submature eggs of both species were semitransparent and soft, but mature eggs near the oviducts had hard shells.

3.3. Vertical Distributions of H. matsumurai Eggs and Duration of Submergence Underwater. Figure 5 shows the vertical distribution of the eggs of *H. matsumurai* laid and the percentage duration of submergence under seawater. The positional height of the eggs ranged from 161 cm to 273 cm with an average of 213 cm. Thus, most eggs were laid above the average sea level (164 cm) at station TW. They were submerged under water at most twice a day. The total duration of submergence from November 2008 to April 2009 ranged from 3.8% to 51.5%.

4. Discussion

4.1. Life Cycles of H. matsumurai and A. shiranui. Our observations in the field indicated that (1) neither nymphs nor adults of *H. matsumurai* or *A. shiranui* were found during winter or early spring; (2) the eggs of *H. matsumurai* were seen throughout the year; (3) young nymphs of both species were first observed in May or June. Older nymphs and adults appeared later (Figures 3 and 4); (4) both species probably overwinter in the egg stage with 1st instar nymphs of the overwintering generation hatching out in May or June.

We estimate that for *H. matsumurai*, nymphal development time from overwintering eggs lasted approximately 40~50 days with the adults appearing in July (Figure 3). After July, composition of developmental stages was rather obscure. There were considerable variations in timing of

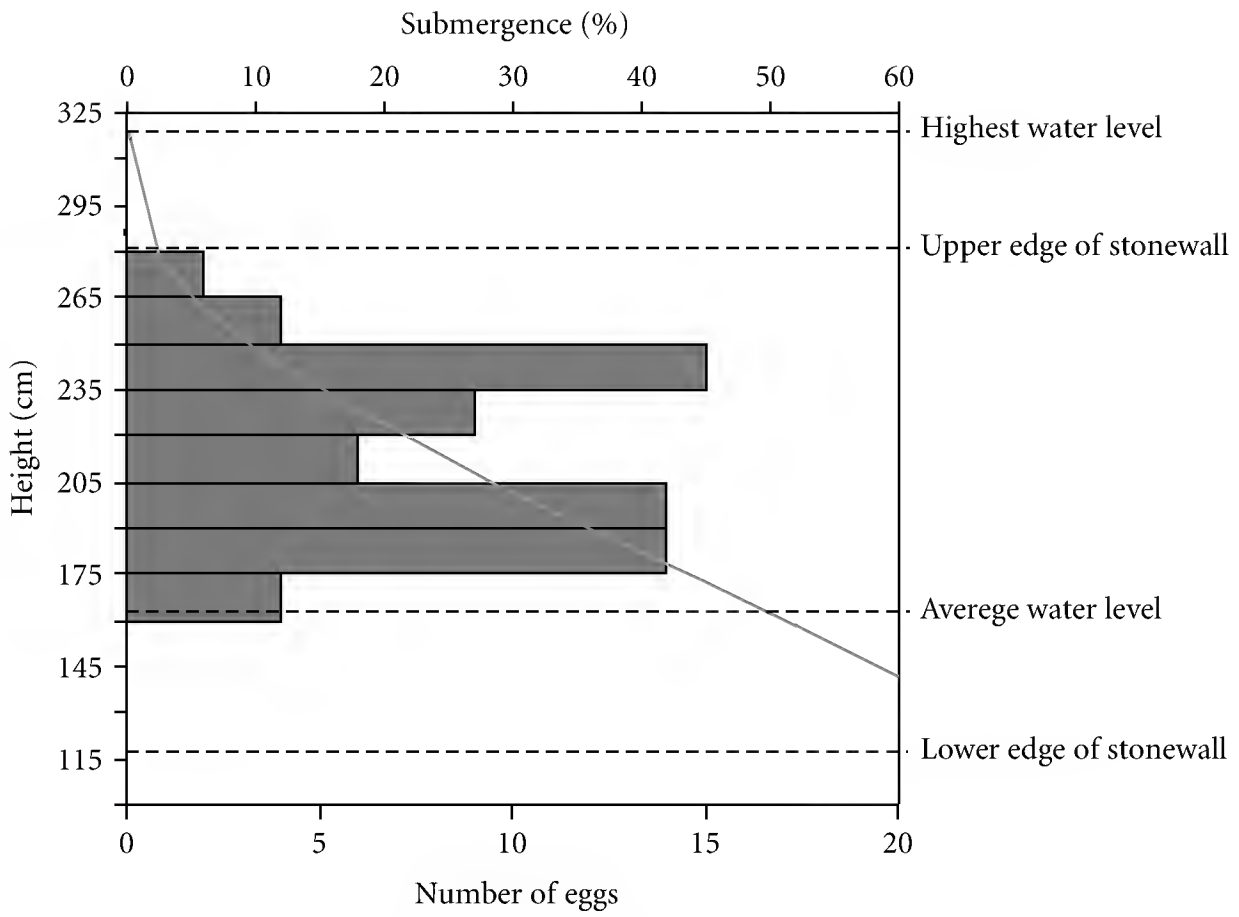

FIGURE 5: Frequency distribution of height of *H. matsumurai* eggs laid on a sandstone wall in relation to tidal level. The solid line is percent of duration of submergence under water of the period from November 2008 to April 2009.

eclosion of eggs, which ranged from early May to late June [Ikawa et al., unpublished observation]. These variations might have led to the concurrent appearance of different stages in later generations. Copulating adults and newly laid eggs were both found in July. Hence, young nymphs found in late July and early August were presumed to belong to the subsequent generation. We found two peaks of young nymphs (in late July–early August and in late August–September), which we assume to belong to two different generations. However, some of the young nymphs that appeared in September would probably die before reaching the adult stage. In October and November, only small numbers of adults were found. Throughout the year, whenever adults were present, most females were found to have mature and/or submature eggs in their ovarioles (Table 1), suggesting that females may lay eggs from July until late autumn. Thus, *H. matsumurai* probably overwinters in the egg stage, the overwintering generation ecloses in May or June, and there may be two or three generations a year.

The life cycle of *A. shiranui* is similar to that of *H. matsumurai* (Figure 4, Table 2). The eggs of *A. shiranui* have not yet been found in the field. However, temporal changes in the appearance of nymphs and adults suggest that it repeats two or three generations a year and overwinters in the egg stage. *A. shiranui* probably also lays eggs on sandstones on the shore since adult females kept in a tank in the laboratory laid eggs around the waterline on the sandstones provided [Nozoe, unpublished observation]. The oviposition site of *A. shiranui* could be very different from that of *H. matsumurai*.

Not much is known previously about the lifecycles of coastal *Halobates* and *Asclepios*. Because the sea-surface temperature at Kujukushima falls below 15°C during the winter, the winter diapause would be a requisite for the sea skaters. Our study suggests that *H. matsumurai* and also probably *A. shiranui* overwinter in the egg stage at Kujukushima. On the other hand, temperate gerrids overwinter most often as adults [26, 27]. Egg diapause in Gerridae is known only for *Metrobates hesperius* [28] and *Metrocoris histro* [29] and was postulated for a few genera [27]. *Metrocoris* as well as *Halobates* and *Asclepios* belongs to the subfamily Halobatinae.

4.2. Egg Load of Adult Females. Average numbers of mature or submature eggs per female of *H. matsumurai and A. shiranu*i were at most 7 or 8 (Tables 1 and 2). These values are similar to those of *H. japonicus*, the other coastal species of *Halobates* found in Japan [20, 21]. The size of *H. matsumurai* egg was slightly larger than that of *H. japonicus* (ca. 1.5 mm long and 0.4 mm wide) [20, 21] and much larger than that of *A. shiranui* (Tables 1 and 2). The egg size of *Halobates* spp. ranges 0.8–1.3 mm long and about 0.5 mm wide [7]. Thus, *H. matsumurai* and *H. japonicus* have relatively larger eggs in the genus *Halobates*. Adults of the two species are morphologically similar [8]. However, the mature eggs in the oviduct of *H. japonicus* are semitransparent and soft without hard eggshell [Ikawa, unpublished observation], while those of *H. matsumurai* and *A. shiranui* have the hard eggshell (the present study). No sea skaters are known to have such hard eggshells as *H. matsumurai* and *A. shiranui.*

4.3. Vertical Distributions of H. matsumurai Eggs. The eggs of *H. matsumurai* were laid on natural sandstones and sandstone walls along the shore, mostly above average water level, and were submerged less than 50% of the time during the egg stage. *H. fijiensis*, the only other coastal *Halobates* for which oviposition site in the field is known, laid its eggs mainly on the leaves of the turtle grass *Syringodium*

isoetifolium or on the fronds of the green algae, *Halimeda* sp. [19]. Both plants are sublittoral and remain submerged underwater most of the time. Oviposition behavior of *H. fijiensis* was observed only during days of extreme low spring tide when these plants were exposed to the air. Foster and Treherne [19] concluded that eggs laid at the extreme low spring level would be protected from overheating and desiccation, and perhaps also from the wave action. They might also be protected from parasitoids. In fact, percentage parasitism of eggs of pond skaters was found to decrease with increasing depth under submersion of eggs (e.g., [30]).

It must be more adaptive for *H. matsumurai* to lay eggs above the average water level than at low water level as *H. fijiensis* does. A possible selective force for *H. matsumurai* to oviposit above the average water level in Kujukushima could be paucity of suitable oviposition sites at low water level. The area around low water mark in Kujukushima is mostly sandy, with neither mangrove roots nor abundant sublittoral plants. In addition, most grass plants along the shore die during the winter. Sandstones on the shore might be the only stable substrate for oviposition. However, the eggs laid on sandstones are exposed to solar radiation, wind, and storms, and also to predation. *H. matsumurai* might have developed the hard eggshell as a protection against such harsh physical conditions, especially during the long overwintering period.

Acknowledgments

The authors thank the members of Kujukushima Aquarium and T. Matsuo for assistance of field survey. They are indebted to A. Chiba, M. Iwabe, and R. Takeya for their help with various laboratory work especially identification of sea skaters and to Sasebo Coast Guard Office for offering the tidal data. Special thanks go to L. Cheng, H. Okabe, Y. Suzuki, and S. Unher for helpful discussions and/or critical reading of the paper. This study was partly supported by the Academic Grant of Morioka University for the year of 2008.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 215891, 5 pages
doi:10.1155/2012/215891

Research Article

Vertically Stratified Ash-Limb Beetle Fauna in Northern Ohio

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Received 29 May 2012; Accepted 9 June 2012

Academic Editor: Martin H. Villet

To better understand the diversity and ecology of indigenous arthropods at risk from the invasive emerald ash borer (*Agrilus planipennis* Fairmaire) in North American forests, saproxylic beetles (Insecta: Coleoptera) were reared from ash (*Fraxinus* sp.) limbs suspended in the canopy, ~10–17 m above the ground, and from those placed on the ground in a mature mixed hardwood forest. In total, 209 specimens from 9 families and 18 species were collected from 30.0 m^2 of limbs. The generalist cerambycid *Neoclytus acuminatus* (Fabricius) was the most commonly captured taxon, followed by an assemblage of four exotic ambrosia beetles dominated by *Xylosandrus crassiusculus* (Motschulsky). Two species largely or entirely restricted to ash, the buprestid *Agrilus subcinctus* Gory and the curculionid *Hylesinus aculeatus* (Say), were collected as well. Although there were no differences in beetle richness, abundance, or density between limb positions, community composition differed significantly. This can be largely attributed to phloem and wood-feeding species (i.e., Cerambycidae and Buprestidae) being more common in the suspended limbs and ambrosia beetles being more numerous on the forest floor. Possible explanations for these patterns are discussed.

1. Introduction

Saproxylic organisms, species directly or indirectly dependent on dying and dead wood, face numerous anthropogenic threats in many parts of the world. The best studied of these include widespread deforestation and fragmentation, the replacement of complex old-growth forests with younger and more simplified stands, and intensive forest management [1, 2]. The ever-worsening invasive species problem poses yet another challenge to this beleaguered fauna. One recent example is the emerald ash borer (EAB) (*Agrilus planipennis* Fairmaire), a phloem-feeding buprestid from Asia responsible for widespread ash (*Fraxinus* spp.) mortality in eastern North America. This pest was first detected in the Great Lakes region in 2002 [3] and is rapidly spreading into new areas after killing virtually all mature ash trees near its original point of introduction. Thus, much like the previous losses of the American Chestnut and Elm, continent-wide extirpation of the ash genus appears possible. With the window of opportunity to understand the ecology of ash in North American forests quickly and perhaps permanently closing, efforts to better understand faunal communities associated with the genus are of interest.

Although ash is thought to support relatively few arthropod species compared to other temperate deciduous tree genera [4], a number of specialist species are recognized. A recent literature review, for example, revealed that at least 43 arthropod taxa native to North America are dependent on ash [5], nine of which feed on ash phloem or wood. Because extensive surveys of arthropods using ash are lacking, however, this is likely an underestimate of the number of species that will be negatively impacted by the loss of ash [5]. The primary aim of this study was to survey the saproxylic beetle community associated with ash in northern Ohio. We specifically focused on small-diameter ash wood (dead branches) suspended in the canopy as the most likely setting

to encounter previously-undocumented species unique to that diameter class (see [6]). We also focused on freshly-killed branches as opposed to older substrates as specialist species (i.e., species more likely to be at "high risk" from EAB invasion) are known to be more strongly associated with dying and recently dead wood [7].

Despite the recent expansion of interest in forest canopy biology in both tropical [8] and temperate regions [9], little is known about the ecology of saproxylic organisms high above the forest floor [10]. Most information available on their vertical distribution patterns, for example, comes from flight-intercept trap surveys [11] or other passive collection techniques. Although suggestive, these data provide no information on host-use patterns in the canopy. Such information can only be gained by sampling directly from dead wood, but very few efforts have been made to do so [12–14]. This is particularly true for species associated with small diameter dead wood, a neglected size class even at ground-level [15].

Forest arthropod communities exhibit a high degree of vertical stratification, even in temperate forests [16], and the insect community associated with dead ash limbs are likely to vary with height above the ground. To test this, we sampled beetles from ash limbs placed on the forest floor in addition to those suspended in the canopy in this study. Few such comparisons have previously been made. One of the first was by Vodka et al. [13] who distributed bundles of <1–12 cm-diameter sections of oak among different settings in the Czech Republic, including some 17–22 m above the ground in a forest canopy. They collected more species of Buprestidae and Cerambycidae at ground level than in the canopy and concluded that sun exposure was a more important determinant than vertical position. Many species appeared to prefer dead wood placed in the canopy but were not found there exclusively whereas those exhibiting a preference for wood on the forest floor were often restricted to that stratum. Ferro et al. [15] also placed bundles of oak twigs either on the ground, suspended 1.5 m above the ground, or propped against tree trunks in Louisiana, USA. Beetle richness was greater in the elevated bundles than those placed on the forest floor.

2. Materials and Methods

This study took place on NASA Glen Research Center, Plum Brook Station in Erie County, Ohio, USA, a 2600 ha property of considerable ecological value to this highly developed region due to its coverage in meadows, forests, and wetlands [17]. One of the most mature tracts of mixed hardwood forests, located near the northern boundary of the property (Figure 1), was chosen for this study. The forest was dominated by tree genera typical of the region, including *Quercus*, *Acer*, *Carya*, *Fraxinus*, and so forth. Although the exact age of the forest is not known, all forests on the property were cleared prior to WWII according to Bowles and Arrighi [17]. Nine large-diameter ash trees were selected in the forest. Although an effort was made to choose only living trees, four of those selected had already been killed by the emerald ash borer. In late April 2010, 18 major limbs (each ~4.7 m in length, see Table 1) were cut from three healthy (i.e., not yet attacked by EAB) ash trees growing along the roadside nearby. One limb was suspended as high as possible (~10–17 m) in the crowns of the nine trees using heavy rope pulled over a limb high above the ground (Figure 2). A second limb was placed directly below the suspended limb at the base of each tree. We returned about three months after the limbs had been distributed. Each limb was cut into 47 ± 4 sections (range 31–80) measuring 50.8 cm in length, bagged, and brought back to the lab. The midpoint diameter of each section was measured to estimate the total bark surface area of the original limb (bark surface area is a more relevant metric than wood volume for early successional faunas primarily restricted to the phloem layer). All the sections from each limb were bundled together and placed in a rearing bag (following [14]) for about 10 months. All emerging beetles were collected and identified to species. Voucher specimens have been deposited in the University of Georgia Collection of Arthropods (UGCA), Athens, Georgia. The Wilcoxon signed rank test was used to compare beetle abundance, density (i.e., abundance/bark surface area), and richness between limbs placed at the two heights. Analysis of similarities was also performed using PAST [18] to compare beetle composition between heights.

3. Results

In total, 209 beetles belonging to 9 families and 18 species emerged from the ash limbs (Table 2). These included various phloem and wood feeding species, fungivores, and predators (Table 2). The cerambycid *Neoclytus acuminatus* (Fabricius) was the most common species collected, accounting for 51% of all specimens. Nearly 18% of the beetles collected belonged to one of four species of exotic ambrosia beetles, of which *Xylosandrus crassiusculus* (Motschulsky) was the most abundant (Table 2). Only two of the species (~11%) were represented by a single specimen, suggesting that the local ash-limb beetle fauna was well sampled. Two of the species collected, the buprestid *Agrilus subcinctus* Gory and the curculionid *Hylesinus aculeatus* (Say), are known to be largely or entirely restricted to the ash genus. The other species have been recorded from several or many other tree genera (Table 2). Based on the Wilcoxon signed rank test, there were no differences in beetle richness ($P = 1.0$), abundance ($P = 0.7$), or density ($P = 0.7$) between limb positions. Community composition, however, varied significantly between positions based on ANOSIM using both density ($R = 0.15, P = 0.02$) and presence/absence ($R = 0.21, P < 0.01$) data.

4. Discussion

By contrast to Ferro et al. [15] who reared 414 specimens and 35 species from bundles of red oak twigs in Louisiana, we collected only 209 specimens and 18 species from ash limbs despite sampling from about seven times as much wood (~30.0 m^2 versus ~4.3 m^2). Although regional

Table 1: Characteristics of the trees from which ash limbs were suspended and of the limbs themselves.

Tree	Tree condition	Tree DBH[a] (m)	Limbs suspended in canopy				Limbs on forest floor		
			Basal diam. (cm)	Length (m)	SA[b] (m^2)	Height[c] (m)	Basal diam. (cm)	Length (m)	SA[b] (m^2)
1	Living	0.60	6.8	4.3	2.6	17.4	7.5	4.0	1.9
2	Living	0.99	6.0	5.9	1.2	13.5	6.7	4.2	1.7
3	Living	0.68	5.4	5.1	1.4	16.8	4.9	4.8	0.9
4	Dead	0.43	6.3	4.4	1.9	10.7	8.5	4.4	2.6
5	Living	0.58	6.6	5.5	1.7	10.6	8.5	4.8	2.6
6	Dead	0.45	6.3	3.8	1.5	11.7	6.3	4.2	1.1
7	Living	0.51	6.1	5.9	1.8	13.9	7.5	6.0	1.3
8	Dead	0.41	6.0	3.6	1.1	13.0	8.1	5.0	2.1
9	Dead	0.57	5.0	4.6	1.1	13.7	5.7	5.1	1.4
	Mean ± SE	0.57 ± 0.05	6.0 ± 0.2	4.7±0.3	1.6±0.2	13.5±0.8	7.1 ± 0.4	4.7±0.2	1.7±0.2

[a]Diameter at ~1.3 m
[b]Total surface area of limb material from which beetles emerged.
[c]Distance between the forest floor and the base of each limb.

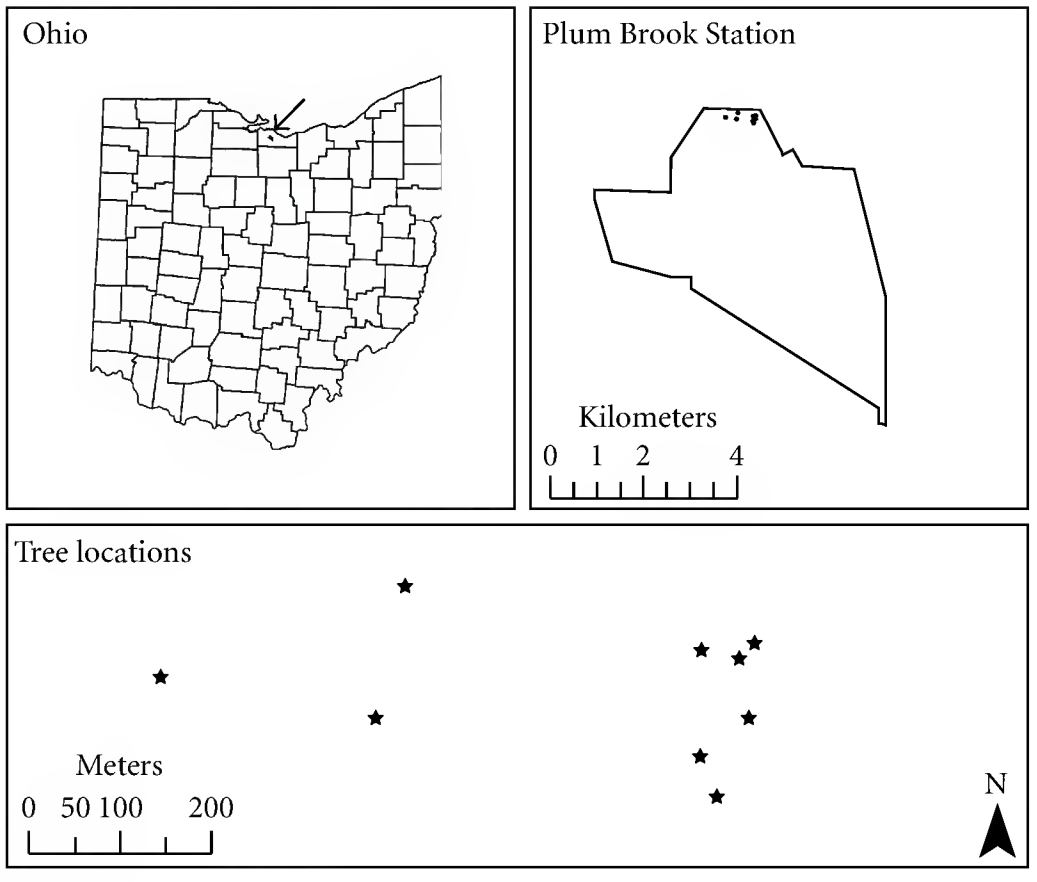

Figure 1: Map of study area in northern Ohio, USA.

(e.g., climate, diversity, etc.) and methodological differences (e.g., waiting longer before sampling) between studies were probably important as well, the small fauna reported herein is consistent with the notion that ash supports relatively few species compared to other tree genera. Such differences among host genera have long been recognized, although previous comparisons are based largely on the foliage-feeding fauna. Elton [19], for instance, remarked that despite "being an old native tree, the ash has a rather thin fauna, as can be ascertained by beating its foliage in the summer. There is none of the rich variety of the oak, nor the aphid biomass of the sycamore." Several possible explanations for differences in insect diversity among tree genera were suggested by Southwood [4]. These include how long a genus has been present in a particular region, its abundance during that time and its diversity and relatedness to other genera present. Compared to oaks, for instance, ash has been less diverse and abundant over time and is more taxonomically isolated from other temperate deciduous tree genera [4].

While most of the beetle species collected in this study are generalists with wide host ranges, two of them are known to be largely or entirely dependent on ash (Table 2). Most notably, *Hylesinus aculeatus* (Say), the eastern ash bark beetle, is restricted to ash and was categorized by Gandhi and Herms [5] as being highly at risk from the emerald ash borer. Although also known to attack *Ligustrum*, *Agrilus subcinctus* Gory is another species at high risk from emerald ash borer. This small buprestid beetle is the only native species of

Table 2: Beetle species collected from ash limbs placed on the forest floor or suspended >10 m above the ground in a temperate deciduous forest. Asterisks indicate nonnative species and degree symbols represent species not previously reported in association with *Fraxinus* according to Gandhi and Herms [5].

Family	Species	Life history	Lower/upper	Total
Buprestidae	*Agrilus subcinctus* Gory	Phloem feeder/wood borer of twigs and branches, restricted to Oleaceae (*Fraxinus*, *Ligustrum*)	0/4	4
	Chrysobothris sexsignata (Say)	Phloem feeder/wood borer, wide host range	1/3	4
Cerambycidae	*Neoclytus acuminatus* (Fabricius)	Phloem feeder/wood borer, wide host range	38/69	107
	Obrium rufulum (Gahan)	Phloem feeder/wood borer, wide host range	0/6	6
Cleridae	*Neorthopleura thoracica* (Say)°	Predator	1/7	8
Corylophidae	*Clypastraea lunata* (LeConte)°	Fungus feeder	2/9	11
Curculionidae: Scolytinae	*Ambrosiodmus rubricollis* (Eichhoff)*°	Fungus feeder (ambrosia beetle), wide host range	4/0	4
	Hylesinus aculeatus (Say)	Phloem feeder/wood borer, restricted to *Fraxinus*	8/2	10
	Hypothenemus eruditus (Westwood)°	Phloem feeder, wide host range	7/0	7
	Xyleborinus saxesenii (Ratzeburg)*°	Fungus feeder (ambrosia beetle), wide host range	0/1	1
	Xylosandrus crassiusculus (Motschulsky)*°	Fungus feeder (ambrosia beetle), wide host range	26/0	26
	Xylosandrus germanus (Blandford)*	Fungus feeder (ambrosia beetle), wide host range	6/0	6
Laemophloeidae	*Cryptolestes ferrugineus* (Stephens)°	Fungus feeder	1/1	2
	Charaphloeus adustus (LeConte)°	Fungus feeder	4/0	4
Passandridae	*Catogenus rufus* (Fabricius)°	Predator	0/3	3
Silvanidae	*Ahasverus advena* (Waltl)°	Fungus feeder	1/2	3
	Cathartosilvanus imbellis (LeConte)°	Fungus feeder	2/0	2
Zopheridae	*Namunaria guttulata* (LeConte)°	Fungus feeder	0/1	1
	Total		101/108	209

Agrilus known to utilize ash. It specializes on small twigs and was only captured in the suspended limbs in this study. It is also noteworthy that four species of exotic ambrosia beetles were collected, with *Xylosandrus crassiusculus* (Motschulsky) being captured in high numbers. Because these species are known to utilize a wide range of hosts and appear to be dominating scolytine communities in Ohio [20] and elsewhere in North America [21, 22], they represent another exotic threat (i.e., through competitive interactions) to native saproxylic species.

Although we found no differences in beetle richness, abundance, or density between the two limb positions, community composition differed significantly due to taxon-specific stratification. For example, phloem and wood-feeding cerambycids and buprestids were exclusively or more commonly captured emerging from the suspended limbs than from those on the forest floor, a finding consistent with the results from a flight-intercept trap survey in Georgia [11]. One possible explanation for this is that wood suspended off the ground or exposed to sunlight (see [13]) tends to be drier and therefore less dominated by fungi. Fernandes and Price [23], for example, concluded that gall-forming insects prefer the xeric conditions of the upper forest canopy due to a decreased incidence of fungal disease and parasitism. The same could be true for many saproxylic species. Conversely, species that depend on fungi are likely to focus their activities closer to the forest floor where conditions are more favorable for fungal growth. Our data on ambrosia beetles are consistent with this expectation as these species were exclusively or more commonly captured emerging from the limbs on the forest floor compared to those suspended high above the ground.

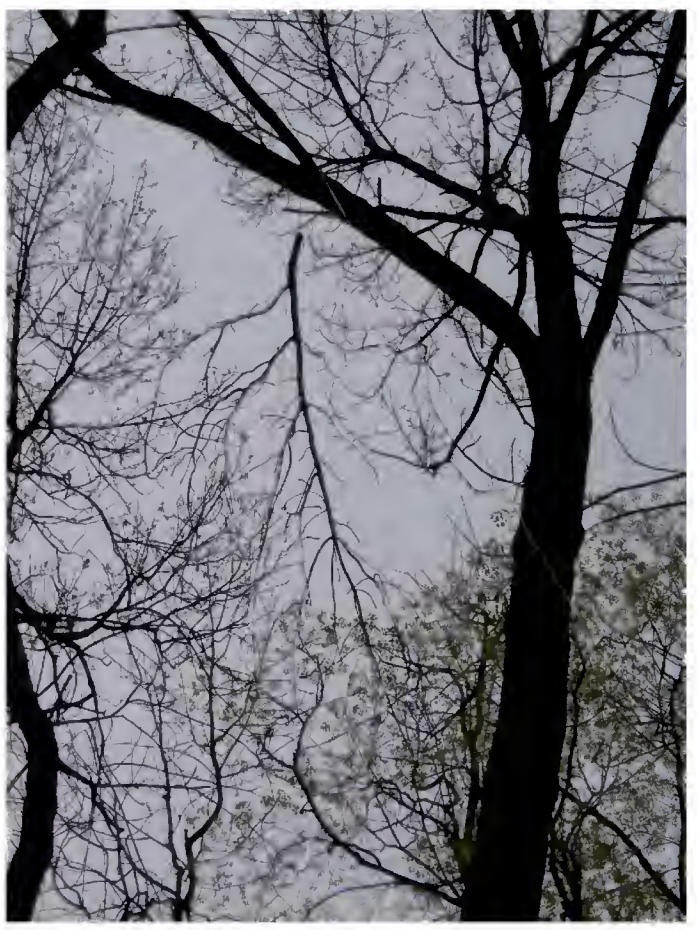

FIGURE 2: An ash limb suspended 17.4 m above the ground.

Acknowledgments

The authors thank Rosemary Giesser and Robert Lallier for facilitating research at the NASA Glen Research Center, Plum Brook Station, and Diane Hartzler, Bryant Chambers, Priya Loess, and Dan Rice for assistance with field work. The authors also thank Mike Thomas for confirming Laemophloeidae identifications and an anonymous reviewer for commenting on an early version of the paper.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 193029, 9 pages
doi:10.1155/2012/193029

Research Article

Effects of Long Distance Transportation on Honey Bee Physiology

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Received 4 April 2012; Accepted 24 May 2012

Academic Editor: Tugrul Giray

Despite the requirement of long distance transportation of honey bees used for pollination, we understand little how transportation affects honey bees. Three trials in three different states (CA, GA, and MI) were conducted to study the effects of long distance transportation on honey bee physiology. Newly emerged bees from one colony were split into two groups and introduced into a transported (T) colony or a stationary (S) colony in each trial. Volumes of hypopharyngeal gland acini in T colonies were significantly smaller than S colonies in all three trials. There were no significant differences between S and T colonies in juvenile hormone titers. Protein content in head showed no significant differences between S and T either in 7-day-old or 17-day-old bees of MI trial, but GA trial showed a significant reduction in bees experiencing transportation. Protein content in thorax was only measured in GA trial and was not significantly different between the two groups. Lipid content in abdomen was not significantly different between the S and T colonies in all three trials. This study suggests that bees experiencing transportation have trouble fully developing their food glands and this might affect their ability to nurse the next generation of workers.

1. Introduction

Honey bees (*Apis mellifera*) are perhaps the most important insects to humans due to their pollination service provided to agriculture [1]. Honey bees experience many different types of stresses. They are impacted by parasitic mites such as *Varroa destructor* and *Acarapis woodi* [2], microsporidian pathogens such as *Nosema apis* and *Nosema ceranae* [3], hundreds of pesticides applied in crops and brought back by foragers [4], as well as pesticides beekeepers applied inside the colonies [5]. Besides these stresses, perhaps the strongest stress experienced by honey bees is long distance transportation. For example, bees are transported from Florida to California, across four time zones in the spring for almond pollination. About 50% of bee colonies in Michigan also migrate to south (e.g., Georgia and Florida) for overwintering and then are moved back for apple and cherry pollination. Yet we understand little of the effects of the long distance transportation on bees because no studies have ever been conducted to determine the physiological or behavioral changes induced by such stress.

Honey bees have an age-related division of labor whereby young workers stay inside taking care of brood (nurses), and old bees forage for food (nectar and pollen) and other resources (water and propolis). This progression of behavioral changes is associated with juvenile hormone (JH), with nurses having low levels of JH and foragers having high ones [6–8]. Although JH is not considered a stress hormone [9], JH titers in blood can tell us whether workers are switching to become foragers or not. JH has been shown to be antagonistic to vitellogenin (Vg) [10], whereby bees with low JH always have high Vg and high JH suppresses Vg. Vg has been shown to be associated with slower aging because it protects workers from oxidative stress [11, 12] and is higher in queens [13], who live longer than workers. Workers that are ready to forage are characterized with high JH, low Vg, low abdomen fat, and lower body protein, essentially becoming "disposable" from the colony's point of view. JH therefore is a reliable indicator of the physiological age of workers. The hypopharyngeal glands (HPGs) of honey bees play a critical role in social

cohesion, because they provide secretions rich in protein, which are fed to larvae of all three castes, and also to adult queen, drones [14, 15], and foragers [16]. The sizes of HPG reflect how good a protein nutrition the bees have obtained prior to becoming nurses and may affect their nursing ability [17, 18] or it can reflect the effect of pathogens such as *Nosema apis* [19]. The amount of abdomen fat is another indicator for when workers become foragers, with nurses having high levels and foragers low levels [20]. Protein levels in workers will tell us whether workers during transportation can still obtain adequate protein nutrition or not. For example, can they still find/eat the same amount of pollen while "on the move" on the truck? Are their digestion efficiency affected by transportation?

In this study, we determined for the first time whether bees undergoing long distance transportation have higher JH levels (aging prematurely) and whether their hypopharyngeal gland sizes, total protein content in head or thorax, and lipid content in abdomen are smaller or lower due to transportation. Our hypothesis was that either due to higher mortality of older bees during transportation or loss of them due to drifting after transportation, or due to inadequate pollen consumption by young bees, we should see higher JH titers, smaller HPGs, lower protein content in heads and thorax, and lower lipid content in abdomen, in the bees experiencing transportation.

2. Materials and Methods

2.1. General Methods. A group of "transported" (T) honey bee colonies were moved to another location (CA trial), or traveled a round trip and returned to the original location (GA, MI trials). A group of "stationary" (S) colonies were not moved and served as the control. About 8–16 days prior to the transportation of bees, newly emerged bees from strong colonies were obtained by incubating sealed brood in make-shift "incubators" (34 ± 2°C, 50% RH, CA and GA trial), or a laboratory incubator (34 ± 1°C, MI trial). The incubating area was in a bathroom with 1-2 space heaters and a thermostat controlled power strip that powered the heater(s). Workers were obtained from a source colony (300–704 per colony), painted with Testor's color paint and split equally into two subgroups. One subgroup was introduced into a T colony, and the other subgroup was introduced into an S colony. Each of the S and T colonies receiving the same group of workers was termed a "colony-pair." This controlled any possible genetic differences among workers [21]. Any differences in measured parameters would be due to the treatment regime (transported or not), because colony differences (amount of food and brood) were controlled by having each colony-pairs as similar as possible in each trial. In each trial, for each total number of colony-pairs ($N = 6$ to 12), we obtained brood from $N + 3$ colonies to insure there were enough newly emerged bees for each colony-pair and did not use bees from the three lowest yielded colonies. Each colony pair therefore received bees from a single source colony.

Bee Bleeding. Hemolymph was taken for JH titer measurement. Sampled workers were bled according to established procedures [22]. Briefly, a small hole was pricked with a bent insect pin between the 4th and 5th abdomen segment of sampled bees. The hemolymph was collected in a capillary tube (Drummond Wiretrol 1 to 5 μL, Drummond Scientific company, USA) and then measured to the nearest 0.5 mm with a ruler and mixed with 500 μL of acetonitrile (EM Science) in a 12 × 125 mm culture tube with a Teflon-lined lid. The length of hemolymph was then converted to volume by knowing the calibrated mark (5 μL) as 27 mm long.

Juvenile Hormone Titer Measurement. Juvenile hormone (JH) was extracted from the hemolymph and assayed with established procedures [9]. Briefly, JH III in the hemolymph sample was extracted with 1 mL hexane (twice), then the pooled hexane was evaporated using a vacuum centrifuge (Speed Vac Plus SC110; Savant Instrument Inc., Holbrook, NY) linked to a condenser (Savant SS21), which trapped the solvent at −98°C. The dried JH in the sample tube was dissolved in 100 μL of methanol, and a 20 μL aliquot (in duplicate) was taken out, dried, and mixed with 200 μL of gel-phosphate-buffered-saline-Tritone (pH 7.3) containing anti-JH antiserum (1 : 14,000 dilution, generous gift from David Borst) and about 10,000 DPM of [10-3H(N)]-JH (Perkin Elmer, 647.5 Gbq/mmol). The mixture was incubated at room temperature for 2 h, and then 0.5 mL of dextran-coated charcoal suspension (Sigma) was added to each sample tube to absorb the unbound JH. This mixture was incubated in an ice-water mixture for 2.5 min and then centrifuged (2000 g for 3 min). The supernatant, which contained bound JH, was decanted into a glass scintillation vial. Liquid scintillation counting was performed using a Packard 2100TR. A standard curve with various amounts (0, 3, 10, 30, 100, 300, 1,000, 3,000, and 10,000 pg) of standard JH (Sigma) was run each day. KaleidaGraph (Synergy Software, PA, USA) was used to generate a standard curve. Five parameters for the standard curve were obtained by using DPM bound as the dependent variable, JH amount (after log transformation) as the independent variable, using nonlinear regression. The five-parameter formula was described in [22]. Excel (Microsoft, USA) was used to calculate the amount of JH in each sample, by reversing the five-parameter formula (solving for JH with known DPM and the five fitted parameters).

Hypopharyngeal Gland Size Measurement. Hypopharyngeal glands were dissected in 0.9% saline under a dissecting microscope (Olympus SZ12, x32) and then photographed by a digital camera (QImaging Go-3). We then used Image-Pro express 6.0 (Nikon, USA) to measure the width and length of five acini for each bee. The volume of each acinus was calculated as 1/6 × 3.14 × length × width [23].

Protein Content in Head and Thorax Measurement. Protein content in head and thorax was measured similar to hemolymph protein [24, 25]. Briefly, the head or thorax (excluding wings and legs) was removed with a pair of

micro-scissor from individual bees and crushed in 500 μL 1 N NaOH using a plastic pestle and incubated overnight. The solution was then vortexed and centrifuged for 5 min at 2000 g. The solution was then diluted 25 times and 10 μL was loaded (in duplicate) to a cell in a 96-cell plate, each cell was then added 200 μL Bio-Rad Protein dye (Bio-Rad, USA) after 4X dilution. The absorbance of the sample was measured at 595 nm using a Molecular Devices Softmax Pro5 Microplate Spectrophotometer. The amount of protein in each sample was calculated by comparing to a standard curve run each day using known amounts of bovine serum albumin (Sigma-Aldrich, USA).

Lipid Content in Abdomen Measurement. Lipid content in abdomen was measured similar to Toth and Robinson [20], using a colorimetric assay. Briefly, the abdomen was cut from individual bees and the internal organs (digestive tract and the sting apparatus) removed, leaving the cuticle with adhered fat body tissue. Each sample was then homogenized in a mechanical homogenizer (Polytron PT 2100, setting of 12) in 2 mL 2 : 1 chloroform : methanol [26] and allowed to extract overnight. The extract was then mixed with 0.5 mL water and centrifuged at 5000 g for 10 min. The top phase (water) was removed and discarded. The organic phase was filtered through glass wool and adjusted to a total volume of 2 mL. A 100 μL subsample of each lipid extract was dried completely, 0.2 mL concentrated sulfuric acid was added, and samples were heated in boiling water for 10 min. Then, 2.0 mL vanillin reagent (0.6% in 85% phosphoric acid) was added to each sample, which was vortexed and dark-incubated for 15 min to allow pink color formation. Absorbance at 525 nm was measured for each sample using a Molecular Devices Spectra Max 190 multiwell spectrophotometer (Sunnyville, CA, USA). A standard curve using known amounts of pure cholesterol was used to calculate lipid amounts. Each lipid sample was measured in duplicates, and average values were used for subsequent analysis.

2.2. Details of Three Trials

California (CA) Trial. Twelve colony-pairs were used; the S and T groups at Bakersfield, CA.

Newly emerged bees from source colonies were obtained and painted on March 12-13th 2008 then equally divided into two groups. One group was introduced into a T colony and the other half into an S colony. Over 6,000 bees were painted and introduced in two days (150 to 344 bees per colony, 24 colonies). The S group stayed in Bakersfield, CA, while the T group was moved to Florida during a 4 day period (March 14–17th) with a total distance traveled as 4,000 km. On March 18th, 6-7-day-old marked workers were sampled with soft forceps by two people (one at FL, another at CA), and placed them on dry ice, stored at -80°C, then shipped to Michigan State University (East Lansing, MI) for analysis.

Ten bees were thawed on ice, and blood removed for JH determination for each colony of 11 colony-pairs ($10 \times 11 \times 2 = 220$ bees) because one colony was lost due to robbing. A previous study has indicated that blood obtained this way showed lower JH titers compared to that of fresh bees, but the differences between nurses and foragers were maintained (Z. Y. Huang and K. Ahn, unpublished data). Ten bees were dissected for each colony of 9 colony-pairs for HPG size measurement ($10 \times 9 \times 2 = 180$ bees). Ten bees were measured in each colony of 4 colony-pairs for lipid content in abdomen analysis ($10 \times 4 \times 2 = 80$ samples).

Georgia (GA) Trial. Twelve colony-pairs were used for a second trial in Boston, GA. Nearly 6,000 bees (200 to 352 bees per colony) were painted and introduced into 24 colonies on April 18th and 19th, 2008 (but only the first cohort of bees, marked a different color, was used for sampling). The T colonies were moved to Sunfield, Michigan (07:00 April 20th to 15:00 April 21st), rested for one day (April 22nd), and then returned to Boston, GA (12:00 April 23rd to 16:30 April 24th), with a round trip of 3,250 km. The bees had opportunity to fly and forage on April 22nd, 2008, while in Michigan.

On April 25th, ten bees (8 days old) were sampled with soft forceps, put on dry ice for hypothermic anesthesia, and bled immediately (within 30 min) in each colony of the 12 colony-pairs for JH determination ($10 \times 12 \times 2 = 240$ bees). The bled bees were then individually labeled and frozen on dry ice, brought to Michigan, then stored at -80°C freezer until analysis. Since some heads were used to protein measurement, we only analyzed bees from 4 colony-pairs ($10 \times 4 \times 2 = 80$ samples) for HPG. Ten bees were analyzed in each colony of 8 colony-pairs for protein content in head and thorax ($10 \times 8 \times 2 = 160$ heads and 160 thoraces). Ten bees were analyzed in each colony of 4 colony-pairs for lipid content in abdomen ($10 \times 4 \times 2 = 80$ samples).

Michigan (MI) Trial. Six colony-pairs were used for S and T groups in East Lansing, MI. Newly emerged workers (192 to 336 bees per colony) were introduced to the colonies after being painted on May 19th of 2008 (1,430 bees) and 29th of 2008 (2,400 bees). These two groups were intended to be sampled as 17- and 7-day-old bees, respectively, on the date of sampling (June 4th). Transportation was conducted by driving the T colonies about 900 km per day (approximately 08:00 to 17:00), with a total of 2,750 km round trip (from June 1st to June 3rd).

On June 4th, paint-marked 7- and 17-day-old bees were collected by using soft forceps, bled, and stored at -80°C JH titers, HPG size, protein, and lipid analysis.

Ten 7- and 17-day-old bees were sampled in every colony of the 6 colony-pairs for JH determination ($10 \times 6 \times 2 = 240$ bees each age). Ten bees were dissected for HPG size measurement from each colony of the 6 colony-pairs ($5 \times 10 \times 6 \times 2 = 120$ samples). Ten bees were sampled in every colony of 5 colony-pairs for protein content in head analysis ($10 \times 5 \times 2 = 100$ heads). Ten bees were measured in each colony of the 6 colony-pairs for lipid content in abdomen ($10 \times 6 \times 2 = 120$ abdomens).

2.3. Statistical Analyses. Juvenile hormone titers were transformed (logarithmic (JH + 1)) to meet the requirements

of parametric analysis. Differences in JH titers, HPG size, total protein content in head or thorax, and abdomen lipid for bees in S and T groups were analyzed by ANOVA by State View (SAS Institute, NC, USA). Each colony-pair was analyzed separately as an independent comparison, but all colonies in each trial were also analyzed together to compare the overall effect of transportation.

3. Results

3.1. JH Titers in Hemolymph

CA Trial. There were no significant differences between the S and T groups, when all 11 colony-pairs were analyzed together by ANOVA (for F and P values, see Table 1), although colony-pairs 2 and 10 showed differences in JH titers when analyzed as two separate single colony-pairs (Figure 1(a)).

GA Trial. There were no significant differences between the S and T groups when all 12 colony-pairs were analyzed together (Table 1), although S showed significantly higher JH titers than T colony in colony-pair 8 (Figure 1(b)).

MI Trial. There were no significant differences between the S and T groups in 7-day-old bees when all 6 colony-pairs were analyzed together (Table 1), although colony-pairs 3, 4, and 6 showed significant differences between the two groups (Figure 1(c)).

In 17-day-old bees, no significant differences were detected between S and T groups when all 6 colony-pairs were analyzed together by ANOVA (Table 1), although the T had significantly higher JH titers than S colony in colony-pair 1 (Figure 1(d)).

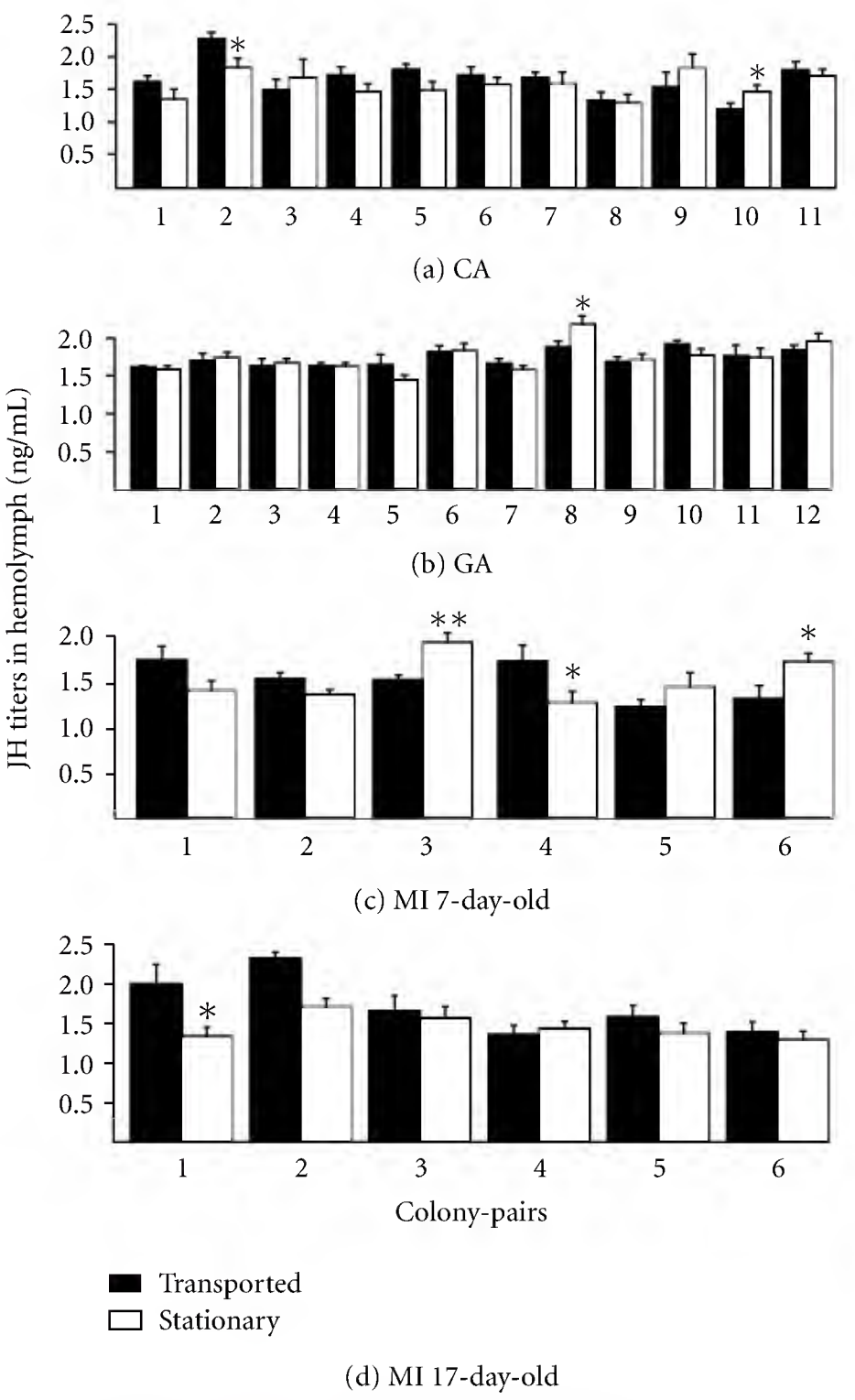

FIGURE 1: Hemolymph juvenile hormone titers (mean ± SE) in worker honey bees experiencing transportation (solid) or no transportation (open) in California (a), Georgia (b), or Michigan ((c), (d)). Workers were 6-7 days old for CA, 8 days for GA and noted in figure for MI. $N = 10$ bees for each colony. Each number represents a colony-pair that hosted genetically similar workers from a source colony. A* inside the open bar indicates that the JH titers between the two colonies within that colony-pair were significantly different (t-test, $P < 0.05$), while ∗∗ denotes highly significant ($P < 0.01$).

3.2. Volume of HPG Acini

CA Trial. There were significant differences in the volume of HPG acini between the S and T groups in the overall analysis (Table 1). If it was analyzed in each colony-pair, six pairs (except colony-pairs 3, 5, and 7) showed significant differences between the S and T colonies in the volume of HPG acini (Figure 2(a)).

GA Trial. Either by overall analysis (Table 1) or each colony-pair (Figure 2(b)), results showed that the volumes of HPG acini in the S group were larger than the T group.

MI Trial. When all colony-pairs were analyzed together by ANOVA, results showed that the volumes of HPG acini were significantly different between the S and T groups (Table 1) in 7-day-old bees. Although in colony-pair 5 the difference was reversed (Figure 2(c)).

In 17-day-old bees, the volume of HPG acini in S groups was significantly larger than T (Table 1), although there were no significantly differences in colony-pair 4, 5, and 6 (Figure 2(d)).

3.3. Protein Content in Head

GA Trial. When all 8 colony-pairs were analyzed together, ANOVA detected a significant reduction in head protein in the T group (Table 1), although only three colony-pairs (1, 3, and 8) showed that S groups had significantly higher protein content in heads when analyzed as single colony-pairs (Figure 3(a)).

MI Trial. For 7-day-old bees, when either analyzed together (Table 1) or as single colony-pairs (Figure 3(b)), there were

Table 1: *F* and *P* values from analysis of variance (ANOVA) conducted for each trial and each parameter, with "ns" denoting nonsignificant ($P > 0.05$). JH titers were transformed (log (JH + 1)) before ANOVA. "Colony source" refers to the differences due to genetic background of source colonies, "transportation" refers to the difference between transported and stationary colonies, and interaction refers to the effect of two (genetics X transportation status). Workers in CA were 6-7 days old, in GA were 8 days old, and those in MI were either 7 or 17 days old.

Item	Trial	Effect	DF	*F*	*P*
JH titers in hemolymph	CA	Colony source	10, 186	5.55	<**0.01**
		Transportation	1, 186	1.70	ns
		Interaction	10, 186	1.77	ns
	GA	Colony source	11, 214	5.98	<**0.01**
		Transportation	1, 214	0.01	ns
		Interaction	11, 214	1.24	ns
	MI 7-day-old	Colony source	5, 106	2.48	**0.04**
		Transportation	1, 106	0.02	ns
		Interaction	5, 106	5.31	<**0.01**
	MI 17-day-old	Colony source	5, 99	2.54	**0.03**
		Transportation	1, 99	6.08	**0.02**
		Interaction	5, 99	1.82	ns
Volume of HPG acini	CA	Colony source	8, 162	6.65	<**0.01**
		Transportation	1, 162	62.65	<**0.01**
		Interaction	8, 162	1.84	ns
	GA	Colony source	3, 72	0.52	ns
		Transportation	1, 72	43.39	<**0.01**
		Interaction	3, 72	0.23	ns
	MI 7-day-old	Colony source	5, 108	8.03	<**0.01**
		Transportation	1, 108	35.44	<**0.01**
		Interaction	5, 108	5.93	<**0.01**
	MI 17-day-old	Colony source	5, 108	5.11	<**0.01**
		Transportation	1, 108	18.17	<**0.01**
		Interaction	5, 108	4.16	<**0.01**
Protein content in head	GA	Colony source	7, 144	17.06	<**0.01**
		Transportation	1, 144	12.96	<**0.01**
		Interaction	7, 144	1.89	ns
	MI 7-day-old	Colony source	4, 90	12.27	<**0.01**
		Transportation	1, 90	1.24	ns
		Interaction	4, 90	0.63	ns
	MI 17-day-old	Colony source	4, 90	1.00	ns
		Transportation	1, 90	6.55	<**0.01**
		Interaction	4, 90	2.91	<**0.03**
Protein content in thorax	GA	Colony source	7, 144	13.10	<**0.01**
		Transportation	1, 144	1.86	ns
		Interaction	7, 144	1.83	ns
Lipid content in abdomen	CA	Colony source	3, 72	2.44	ns
		Transportation	1, 72	0.02	ns
		Interaction	3, 72	4.60	<**0.01**
	GA	Colony source	3, 72	10.54	ns
		Transportation	1, 72	2.47	ns
		Interaction	3, 72	5.09	<**0.01**
	MI 7-day-old	Colony source	5, 108	4.84	<**0.01**
		Transportation	1, 108	2.09	ns
		Interaction	5, 108	1.54	ns
	MI 17-day-old	Colony source	5, 108	1.42	ns
		Transportation	1, 108	2.53	ns
		Interaction	5, 108	1.27	ns

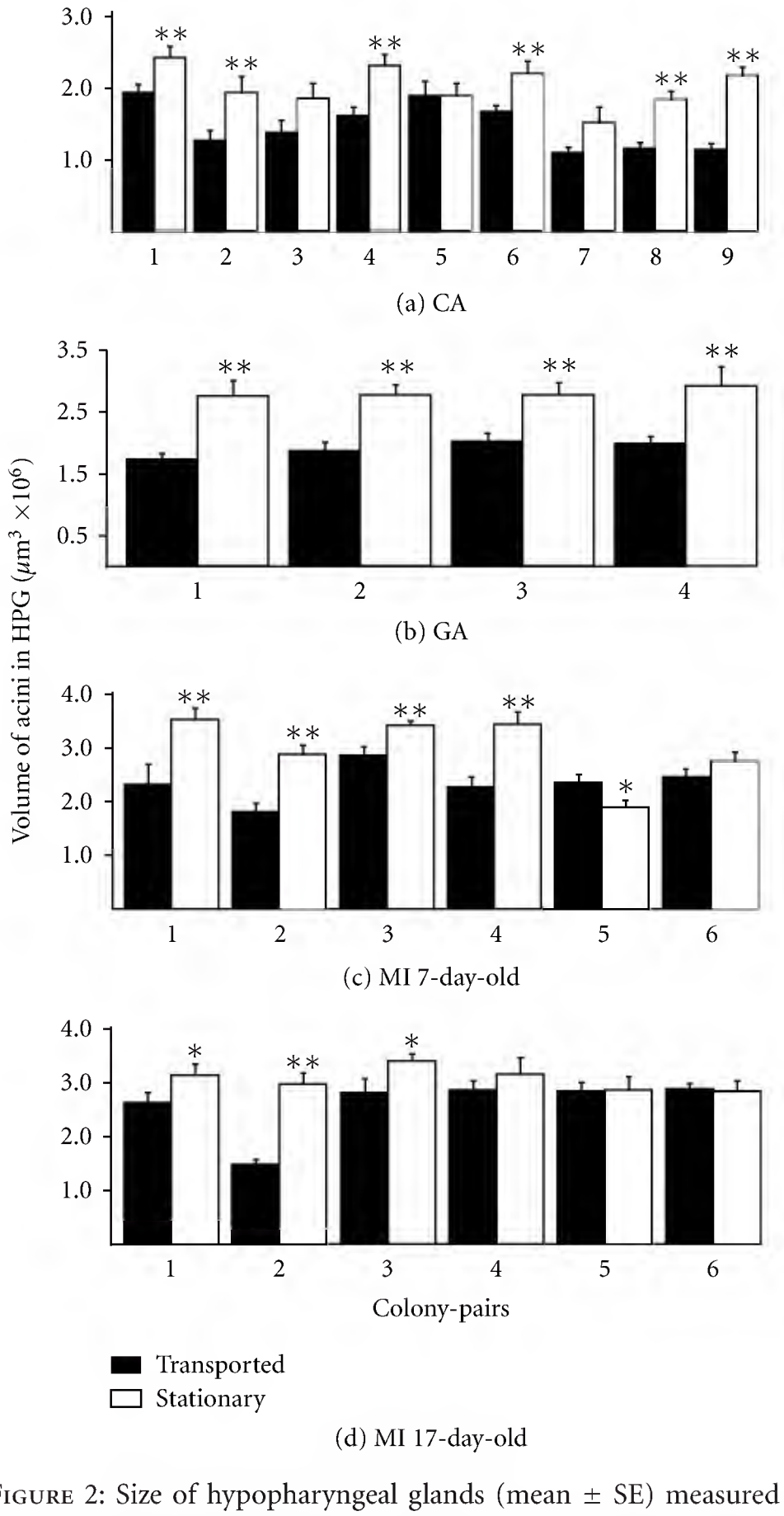

FIGURE 2: Size of hypopharyngeal glands (mean ± SE) measured as volume of gland acini in worker honey bees experiencing transportation (solid) or no transportation (open) in California (a), Georgia (b), or Michigan ((c), (d)). For more details, see Figure 1 legend.

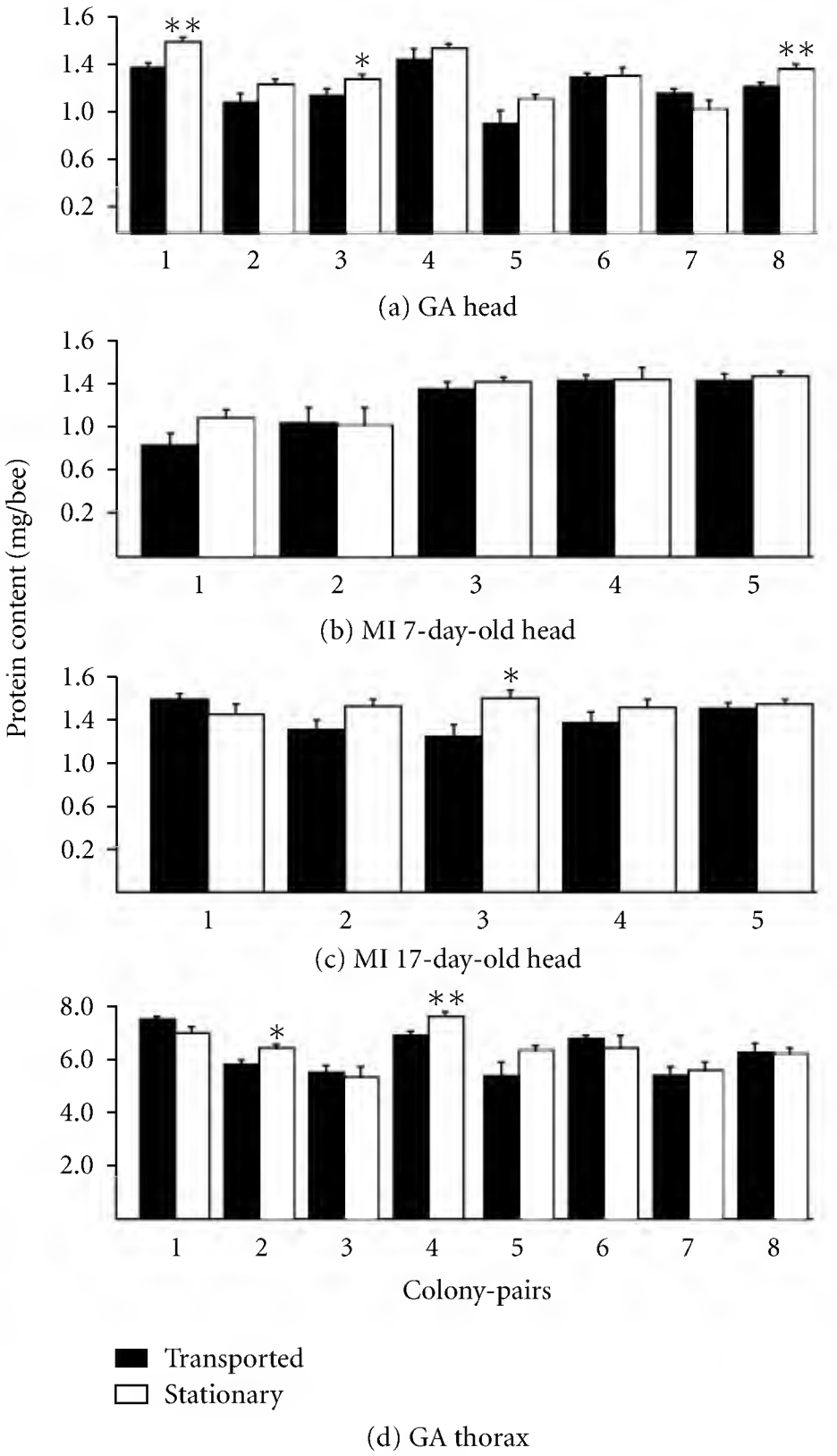

FIGURE 3: Protein content (mean ± SE) in worker honey bees experiencing transportation (solid) or no transportation (open) in heads ((a), (b), (c)) or thorax (d). For more details, see Figure 1 legend.

no significant differences between the S and T groups in 7-day-old bees.

For 17-day-old bees, there were no significant differences when analyzed together (Table 1), although colony-pair 3 showed significant differences between S and T (Figure 3(c)).

3.4. Protein Content in Thorax

GA Trial. There were no significantly differences between S and T groups in thorax protein content when analyzed together (Table 1), although the S colonies were significantly higher than T colonies in colony-pairs 2 and 4 (Figure 3(d)).

3.5. Lipid Content in Abdomen

CA Trial. There were no significant differences between the S and T groups (Table 1), although colony-pair 3 showed a significant difference between the two groups (Figure 4(a)).

GA Trial. There were no significant differences detected between S and T group when all 4 colony-pairs were analyzed together (Table 1), although S had higher lipid content in abdomen than T in colony-pair 1 (Figure 4(b)).

MI Trial. For 7-day-old bees, there were no significant differences between S and T groups in lipid content in abdomen (Table 1), individual pairs also did not show any differences (Figure 4(c)).

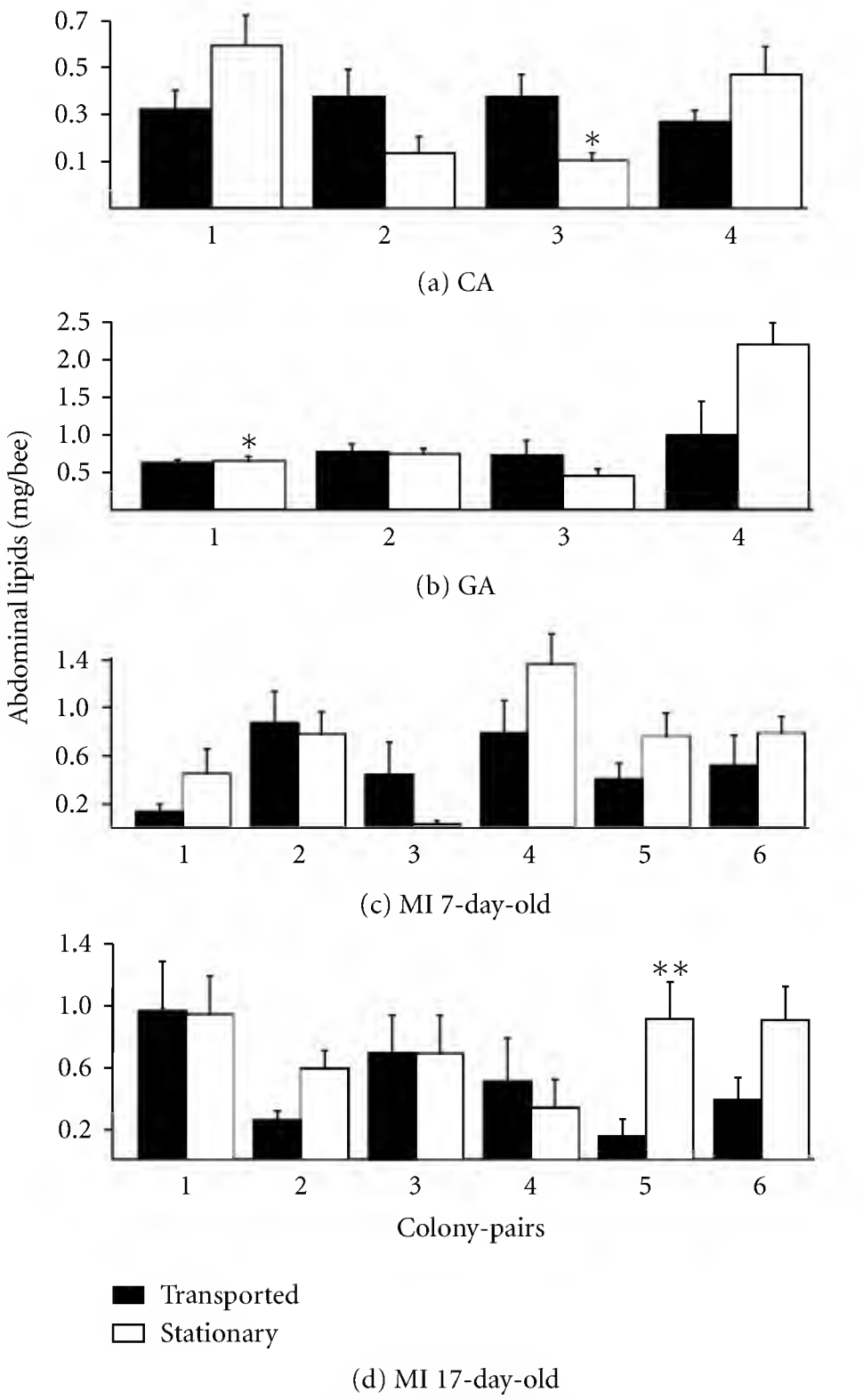

FIGURE 4: Lipid content (mean ± SE) in worker honey bees experiencing transportation (solid) or no transportation (open) in California (a), Georgia (b), or Michigan ((c), (d)). For more details, see Figure 1 legend.

For 17-day-old bees, there were no significant differences between S and T groups when all 6 colony-pairs were analyzed together (Table 1), although S had higher lipid content in abdomen than T (Figure 4(d)).

4. Discussion

The major finding from this study was that HPG sizes were consistently and negatively affected by transportation. Results showed that the volume of HPG acini were significantly smaller in bees from transported colonies than that from stationary ones (Figure 2). This was true for all three trials conducted in different locations, and also for both young (7-day-old) and old (17-day-old) bees in the Michigan trial. Previous studies have shown that consumption rate of protein diets had a positive correlation with the development of HPG [27, 28]. In this study, the worker bees in T group showed consistently smaller HPG sizes, possibly due to their inability to find or consume pollen normally. It is also possible that trophallaxis was adversely affected such that the initial flow of jelly to very young bees (1–4 days old) from nurses occurred at a lower frequency such that normal HPG development was affected [16]. Yet another possibility is that the queen stopped laying during transportation and the overall level of brood pheromone would be lower in the transported colonies, and this could have affected their HPG development negatively. It is a bit surprising that 17-day-old bees were also affected in their gland size, because these bees were 13 days old when the transportation took place in the MI trial. Workers should have attained their maximum gland size around 12–14 days old [29], however, transportation during this time period still significantly negatively impacted their gland size. It is possible that these 17-day-old bees were actively nursing brood during transportation but they were unable to balance their protein input with proper pollen feeding. This suggests that all in-hive workers (workers that are performing preforaging duties) are affected by transportation. It is puzzling that while HPG acini sizes showed consistent differences in all trials, the head protein or thorax protein content did not show the same pattern. Head protein includes HPG and brain, plus head salivary glands and muscles for the mandibles. Our data here suggest that HPG size responded more consistently to transportation stress. Thorax protein content mainly reflects the mass of the flight muscles, for some reason it also does not show a consistent effect. It is possible that HPGs respond more rapidly, but changes in head or thorax protein content have more variability and do not show the same sensitivity to stress-related events. A recent study also failed to find any differences in bees from healthy and colonies exhibiting colony collapse disorder (CCD) in weights or protein content of head, thorax, abdomen [30].

JH is well studied because it plays many roles in honey bees. It has been known to be involved in the queen-worker caste differentiation during the larval stage [31, 32], in regulating the age-related division of labor in adult workers [33], and in determining aggression levels in workers [34]. JH titers are regulated by the changes in rate of JH biosynthesis and other processes, such as degradation and tissue uptake [35]. A previous study showed that JH levels in foragers also displayed diurnal changes. JH titers were at their lowest just before noon, slightly increased by late afternoon and peaked just before midnight [36]. Lin et al. provided the first evidence that JH titers changed rapidly when workers were removed from their normal social environment and manipulated experimentally [9]. Therefore, JH titers in hemolymph of honey bees were influenced by many factors.

In this study, there were no differences in JH titers between S and T groups, when all colony-pairs were analyzed together by ANOVA. This is contrary to our original hypothesis that transported bees should have higher JH titers than stationary bees. It is possible that our bees were too young ($\leq$8 days in all trials except MI 17-day-old bees) to observe an effect on JH. However, even in 17-day-old bees (Figure 1(d)), only colony-pair 1 showed significantly higher

JH titers in the T group, while colony-pair 2 showed a trend but it was not significantly different. It is possible that JH is affected by too many factors and is not a good indicator for transportation-related stress. Alternatively, the transported bees did not experience a faster behavioral development as we originally hypothesized.

Toth and Robinson found that abdominal lipid stores in honey bees decline prior to the onset of foraging [20]. Before this study, we had hypothesized that lipid content in abdomen should be lower in transported bees, either due to faster behavioral development, or due to less pollen consumption during the long distance transportation. However, the data here showed that there were no significant differences between stationary and transported groups in their fat content. This agrees with the JH data, suggesting that transported bees did not experience a faster behavioral development.

Our study concentrated on younger bees in the colony, assuming that they would be more sensitive to transportation-generated disturbances. However, it might be that the older bees are more sensitive to this process. Foragers, for example, might experience higher mortality due to their higher metabolism, and also due to a lack of jelly fed to them during the transportation, as suggested by this study. We assumed that the physiological responses were maximal immediately after the transportation took place. However, it is possible that it might take 3-4 days for the effect to be manifested and we might therefore have missed effects on accelerated development. We also did not know how long the negative impact lasted on the transported bees or whether their glands would recover after another week. A laboratory proxy for long distance transportation is needed to further dissect the detailed mechanisms of transportation-induced stresses.

Acknowledgments

This project was funded by grants from North American Pollinator Protection Campaign (NAPPC), National Honey Board (NHB), Generating Research and Extension to meet Economic and Environmental Needs (GREEEN) at the Michigan State University, USDA NIFA AFRI "Managed Pollinator Coordinated Agricultural Project" (2009-85118-05718) (to Z. Y. Huang), and the USDA-ARS Areawide Program to improve bee health (to J. Pettis). The authors thank Nathan Rice, Frank Eischen, Dennie Best, and Oren Best for their help in the course of this project and anonymous reviewers for suggestions on this paper.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 975069, 9 pages
doi:10.1155/2012/975069

Research Article

Entomopathogens Isolated from Invasive Ants and Tests of Their Pathogenicity

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Received 5 April 2012; Revised 20 June 2012; Accepted 21 June 2012

Academic Editor: Ai-Ping Liang

Some ant species cause severe ecological and health impact in urban areas. Many attempts have been tested to control such species, although they do not always succeed. Biological control is an alternative to chemical control and has gained great prominence in research, and fungi and nematodes are among the successful organisms controlling insects. This study aimed to clarify some questions regarding the biological control of ants. Invasive ant species in Brazil had their nests evaluated for the presence of entomopathogens. Isolated entomopathogens were later applied in colonies of *Monomorium floricola* under laboratory conditions to evaluate their effectiveness and the behavior of the ant colonies after treatment. The entomopathogenic nematodes *Heterorhabditis* sp. and *Steinernema* sp. and the fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces* sp. were isolated from the invasive ant nests. *M. floricola* colonies treated with *Steinernema* sp. and *Heterorhabditis* sp. showed a higher mortality of workers than control. The fungus *Beauveria bassiana* caused higher mortality of *M. floricola* workers. However, no colony reduction or elimination was observed in any treatment. The defensive behaviors of ants, such as grooming behavior and colony budding, must be considered when using fungi and nematodes for biological control of ants.

1. Introduction

Among social insects, some ant species have adapted to disturbed environments in urban areas. In Brazil, it is estimated that from 2000 identified species of ants, 50 species are pests causing losses in rural and urban areas and damage to public health [1]. Some of them, native or exotic to the country, have the status of invasive species.

Biological invasion occurs when a species has competitive advantage, natural obstacles to deter their proliferation are not present, disperses quickly, and invades new areas, becoming a dominant population [2]. In such cases, control measures must be defined in order to minimize problems associated to the invading species. Integrated pest management (IPM) has been used as rational pest control practices and biological control is among the several used tools [3]. The recognition of biological control agents in the field is essential for successful use of exotic natural enemies [3] such as entomopathogens, parasitoids, and predators [4].

Each natural enemy offers advantages for the control of different pests, but some of them are better used on urban pests, such as the entomopathogens, including fungi and nematodes. Human tolerance to these natural enemies must be considered [4]. Therefore, when the problem faced is at urban ant species, caution is advised.

Ant behavioral adaptations can cause failures of control measures using natural enemies. Thus, an understanding of ant defenses is necessary when biological control measures are used [5]. Several studies have been conducted in order to test different natural enemies of ants, especially on the Brazilian native fire ants, *Solenopsis invicta* [6–12]. In order to contribute to the searches of natural enemies of ants and

their effects on these insects, this study evaluated the presence of entomopathogenic fungi and nematodes inside ant invasive species nests and their pathogenicity compared to entomopathogenic fungi deposited in a scientific collection.

2. Materials and Methods

2.1. Entomopathogens Isolated from Ant Invading Species Nests in Brazil. Soil sample was collected from invasive ant nests and from the soil three meters away from them (control soil samples). Samples were taken from 10 to 15 cm deep in the soil, set in plastic bags, and processed in the laboratory. Ants were discarded.

Collections were made in 70 localities in the states of São Paulo, Minas Gerais, Rio Grande do Sul, Mato Grosso do Sul, and Rio de Janeiro. Samples were conducted in those places where invading species were present. In this way, collections were not equally distributed in all states.

2.2. Entomopathogenic Nematode Isolation. Soil samples were individually placed in vials (600 mL) with five *Galleria mellonella* (Lepidoptera: Pyralidae) larvae that were used as baits for the entomopathogenic nematodes possibly present in the samples. Water was added into the vials in order to keep enough moisture for the nematodes reach the larvae. Vials were closed and kept under environment temperature and 12-hour photophase.

After one week, dead *G. mellonella* larvae were transferred to White traps [13] in order to isolate and identify the nematodes.

2.3. Entomopathogenic Fungi Isolation. One gram of the same soil samples for nematode isolation was separated to isolate entomopathogenic fungi. An aliquot of water corresponding to 100x the soil weight was added and the solution was stirred on a Vortex. Serial dilutions were prepared to obtain a 1 : 100 dilution. From the 10^{-2} dilution, 0.1 mL was removed in a biological safety cabinet and inoculated on the surface of a selective medium in a Petri dish and spread with a Drigalsky handle. The Petri dishes were incubated inverted at 27°C ± 1°C. The growth and sporulation of the fungi were assessed at 7 and 15 days after inoculation. Once the presence of fungi in the Petri dish was confirmed, it was transferred to another Petri dish containing potato dextrose agar medium in order to ensure isolation of the pathogen free of contaminants.

Isolated fungi were deposited as pure conidia, in 1.5 mL Eppendorf tubes, and placed in freezer at −12°C for later application on ant colonies for efficacy tests. The isolated fungi were deposited at the Entomopathogenic Collection "Oldemar Cardim de Abreu" from Instituto Biológico, Campinas, Brazil.

2.4. Susceptibility of Colonies of Monomorium floricola to the Entomopathogens in Laboratory Conditions. Colonies of *M. floricola* (Hymenoptera: Formicidae) maintained at the Instituto Biológico Insectarium were used in bioassays to assess their susceptibility to entomopathogens isolated previously and to the entomopathogens deposited in the Entomopathogenic Collection "Oldemar Cardim de Abreu." *M. floricola* was chosen for the control bioassay once it is one of the main tramp ant species in Brazil and because its rearing methodology is well established in the laboratory.

2.5. Colonies of Monomorium floricola. Colonies used for nematode and fungus bioassays consisted of nest cells prepared with Petri dishes (5 cm diameter and 1 cm deep) with the covers painted in black to reduce the intensity of light. Lateral openings were provided to permit ant entrance and exit. Petri dish bottoms were filled with plaster to form a cavity in the center. Two to three thousand ant workers, at least five ergatoid gynes (in *M. floricola* intermediate forms between worker and queen, with a spermatheca, typically substitute the queens for reproduction) and ca. 3 cm^2 of brood (eggs, larvae, and pupae) were placed into the Petri dish. The colonies were then individually placed in the center of a plastic tray (30 × 26 × 8 cm), coated with Teflon 30 (Dupont) to avoid ant escaping. Colonies were maintained in a controlled room (25 to 28°C and 12-hour photophase), fed three times a week with *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae, water, and water/honey (1 : 1).

2.6. Susceptibility to Entomopathogenic Nematodes

2.6.1. Tested Nematodes. Tested nematodes are listed in Table 1 and belong from the Entomopathogen Collection "Oldemar Cardim de Abreu" from the Biological Institute, Campinas, Brazil. Such nematodes have been already tested as biological control for insects of agricultural importance, with established efficacy, but their use as biological control for urban pest ants was still unknown.

Nematodes isolated from ant nests were not used in the bioassays, because we could not rear them in laboratory conditions. They were sensible to manipulation and we did not have success in propagating any of them in vitro.

2.6.2. Bioassays. Five *M. floricola* colonies were used for each nematode isolate, besides the control group, totalizing 15 colonies. The nematodes were prepared in an aqueous suspension containing 5,600 infective juveniles (IFs)/mL (this concentration was higher than that for applications in other insect pests). An aliquot of 2 mL of the suspension was applied with a pipette directly on the colony. Mortality assessments were made daily during the first ten days, and three times a week until the end of the bioassays (33 days). Dead ants were removed with a brush and placed in Petri dishes with moistened filter paper and immediately stored in B.O.D. chamber at 25°C for confirmation of the cause of the death. In the case of confirmed nematode infection, these nematodes would leave the body of ants and would be found on the filter paper. The emerging nematodes were collected and deposited in plastic cups with a thin layer of water and placed in B.O.D. chamber at 18°C before identification.

The general conditions of the treated colonies were evaluated weekly using the same methodology proposed by Jacob [14], including rating the amount of brood, observations on

Table 1: Nematodes belonging to the Entomopathogen Collection "Oldemar Cardim de Abreu" Instituto Biológico, Campinas, Brazil.

Isolate	Nematode	Origin	Collection site
IBCB 02	*Steinernema carpocapsae*	Soil sample	Flórida, EUA
IBCB 24	*Heterorhabditis amazonensis*	*Sphenophorus levis* larvae	Piracicaba, SP

which immature forms were present, presence of alive brood and males, number of ergatoid gynes, and the size of the colony. Such observations were recorded using the following scales: for the amount of brood a value 0 represented absence of brood; 1 little brood; 2 for medium amount of brood, and 3 for large amount of brood present, compared to the initial 3 cm^2 of brood. Types of immature forms were evaluated separately, due the importance of a healthy brood complement in growing colonies and to better characterize the effects of entomopathogens on the colonies. Colonies received a rate of N if brood was absent, E if egg was present; L if larvae were present and P if pupae were present. For quantitative analysis of brood presence, colonies were rated as 0 if brood was absent; 1 if only one immature life stage was present; 2 if two immature life stages were present and 3 if all three immature life stages were present.

The presence (1) or absence (0) of reproductive brood, males and ergatoid gynes were also rated because it provides important information on colony health. The production of sexual forms represents an attempt to perpetuate the colony when conditions become unfavorable.

Colony size was rated as 3 for colonies with normal sizes; 2 for colonies with small size; 1 if colonies showed large decrease in comparison to control colonies; 0 if complete colony death was observed.

2.7. Susceptibility to Entomopathogenic Fungi

2.7.1. Tested Fungi. The fungi selected for testing are shown in Table 2. The isolates from the Entomopathogen Collection "Oldemar Cardim Abreu" were selected because of known efficacy in controlling other pests and for having been isolated from other insects, not ants. The other species of fungi were isolated from invasive ant nests sampled in this study.

2.7.2. Bioassays. As in the bioassay with nematodes, five colonies of *M. floricola* were used for each combination of conidial concentration of fungal isolate, and for the control group, with a total of 65 colonies.

The fungi were grown on Petri dishes containing potato dextrose agar medium [15]. After inoculation, Petri dishes were transferred to B.O.D. chamber at 27°C and 12-hour photophase for a period of 12 days. After this period, the conidia were removed by scraping with a nickel-chromium loop and suspended in sterile distilled water containing the spreading agent (Tween 80) at 0.1%. Conidia were counted using a Neubauer chamber under an optical microscope Zeiss, with 40x magnification.

Dilutions were made to obtain the concentrations normally applied on other insect bioassays (1.0×10^7 and 1.0×10^8 conidia per mL). Each tested colony received 1 mL of each fungi concentration. The fungi were applied with a manual sprayer designed for small surfaces and mounted at a height of 20 cm from the colony. During fungal application, all individuals (ergatoid gynes, workers, and brood) were sprayed by a fine mist of the conidial suspension with entomopathogenic fungi. Control colonies received the similar application, but with water only.

Mortality and colony health evaluations were conducted as for the nematode bioassay, including the weekly evaluations for colony general conditions. Dead ants were removed from the colony tray with the help of a moistened brush, placed in Petri dishes with a wet piece of cotton, and placed in a B.O.D. chamber at 15°C for 15 days for cause of death confirmation. If fungal growth was observed exiting the dead ant bodies infection was confirmed.

2.8. Data Analysis. Data on presence of entomopathogens in nest and control soil samples were compared. Each one of the 70 collection pairs (nests × control soil samples) was analyzed and four possible outcomes were recorded: (a) isolation in either nest or control soil sample, (b) entomopathogens only in the nests, (c) entomopathogens only in the control soil samples, and (d) entomopathogens in both samples. From these four outcomes, we could analyze if nematodes or fungi were present equally using the McNemar Test. This analysis was done separately for fungi and nematodes and later for each isolate. Isolated nematode and fungus species and their respective frequencies were analyzed by the Exact Fisher Test (for samples lesser than five) and by the Pearson Qui Square Test.

In order to analyze *M. floricola* mortality in the laboratory bioassays an analysis of variance with repeated measurements (ANOVA) was used, followed by the post doc Tukey Test for multiple comparisons among the groups, and the Profile Test for time comparisons. The rank transformation was used to obtain variance homogeneity and normal distribution. A 0.05 significance level was used for all statistical tests.

3. Results

3.1. Entomopathogens Isolated from Invasive Ants and Tests of Their Pathogenicity. This work reports for the first time the isolation of entomopathogens inside *Linepithema* spp., *Nylanderia fulva* and *Brachymyrmex pictus* nests. We also isolated entomopathogens from *Solenopsis* spp. as other authors have already registered.

From 70 samples, only 61 were used for analyzing the collected data, once the McNemar test only permits analysis with paired samples (nest and soil control samples).

Table 2: List of tested fungi on *Monomorium floricola* colonies under laboratory conditions.

Isolate	Fungi	Origin	Collection site
Bb 66[1]	*Beauveria bassiana*	*Hypothenemus hampei*	São José de Rio Pardo (São Paulo)
Ma 425[1]	*Metarhizium anisopliae*	Larvae	Iporanga (São Paulo)
P 405[1]	*Paecilomyces* sp.	Larvae	Espiro Santo do Pinhal (São Paulo)
Bb 25	*Beauveria bassiana*	*Solenopsis* sp. nest	São Paulo (São Paulo)
Ma 16	*Metarhizium anisopliae*	*Solenopsis* sp. nest	Corumbá (Mato Grosso do Sul)
P 40	*Paecilomyces* sp.	*Solenopsis* sp. nest	São Paulo (São Paulo)

[1] Isolates from the Entomopathogen collection "Oldemar Cardim de Abreu" from Instituto Biológico, Campinas, Brazil. IBCB 66 = Bb66, IBCB 425 = Ma 425, and IBCB 405 = P 405.

Thus, samples that were accidentally lost led to removal of the paired sample from the analysis. A greater number of entomopathogens was isolated from soil control samples, 49.2% (3), compared to 34.4% ant sample nests (21) ($P = 0.013$).

Higher nematode frequency was found inside *Linepithema* spp., *B. pictus* and *N. fulva* when they were grouped together (4 nests = 30.8%) compared to *Solenopsis* spp. alone (1 nest = 1.79%). Nematodes were not found in 98.2% (55) *Solenopsis* spp. nests and 69.2% (9) other ant species nests grouped together. The Exact Fisher Test showed significance for nematode isolated from the group of other species then *Solenopsis* spp. ($P = 0.004$).

Similar results were recorded for the isolated fungi. A higher frequency of fungi isolated from grouped species was found (8 nests = 72.7%) compared to *Solenopsis* spp. (14 nests = 27.5%), ($P = 0.012$).

3.2. Nematode Isolation. The only nematode species isolated from ant nests, corresponding to 7.3% of the total samples, was *Steinernema* spp. On soil samples *Heterorhabditis* and *Steinernema* were isolated, with a higher frequency of *Steinernema* sp., 8.7%. The frequency of *Heterorhabditis* sp. was 2.9%. Nematodes were not found in 88.4% of the samples.

Occurrence of nematodes inside ant nests and in soil control samples was low. Unfortunately the limited data do not allow complete picture of the role of nematodes inside ant colonies.

From the 70 original samples, two were accidently lost. In this way 68 paired samples were used for analysis. The data seem to show more nematodes on control samples (7.4%) than inside nests (1.5%) but the McNemar test revealed no significant difference between the two samples ($P = 0.103$).

3.3. Fungi Isolation. Three fungi species (*B. bassiana, M. anisopliae*, and *Paecilomyces* sp.) were found both inside ant nests and in the soil control samples. In some samples more than one fungi species was isolated (Table 3). From the 61 evaluated samples (nine were accidently lost), significant difference was found between the number of isolated entomopathogenic fungi inside ant nests (3.3%) and control samples (16.4%), ($P = 0.021$) McNemar Test. *B. bassiana* and *M. anisopliae* were found inside ant nests as frequently as in soil control samples ($P = 0.096$). *Paecilomyces* sp. was more common in soil control samples than inside ant nests ($P = 0.021$) (Table 4).

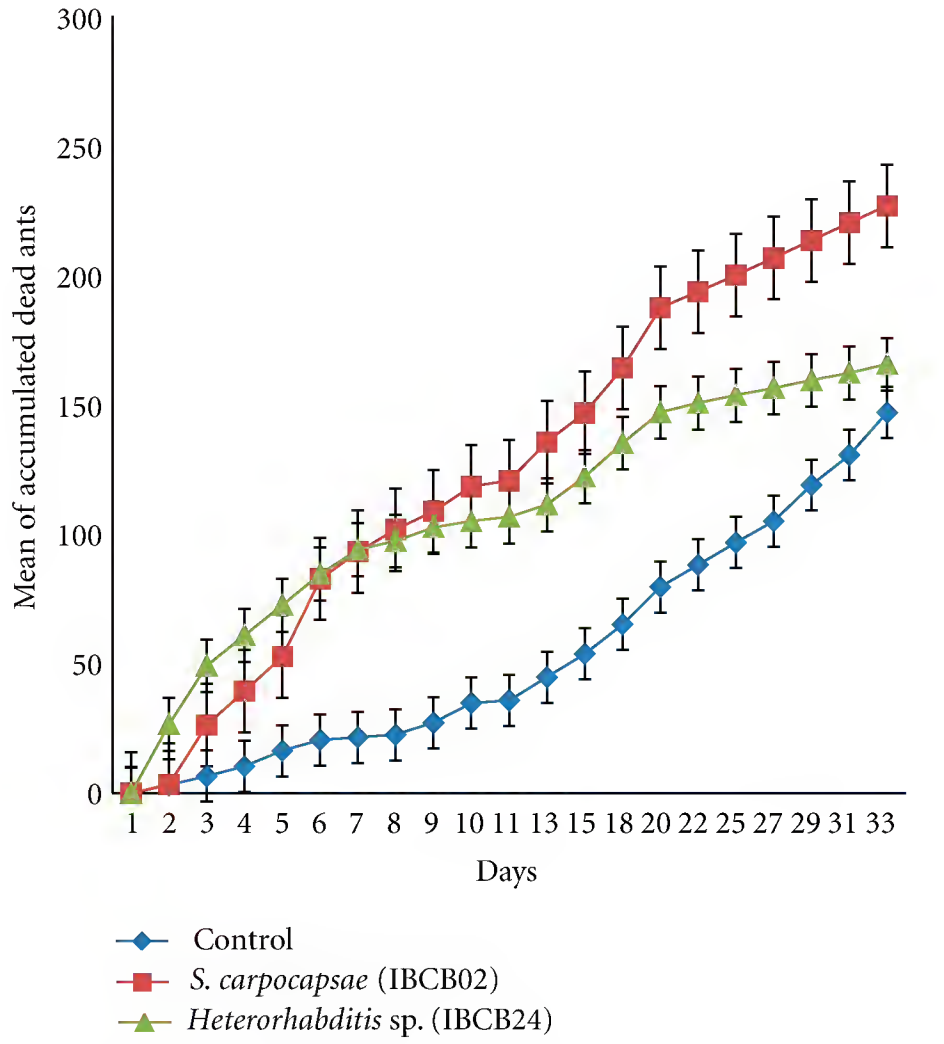


Figure 1: Mean of cumulative mortality of *Monomorium floricola* workers treated with entomopathogenic nematodes from five experimental colonies.

3.4. Susceptibility of Monomorium floricola to Entomopathogens in Laboratory Conditions

3.4.1. Susceptibility to Entomopathogenic Nematodes. *S. carpocapsae* (IBCB 02) and *Heterorhabditis* sp. (IBCB 24) caused *M. floricola* worker mortality (Figure 1). The Tukey Test showed that ant worker mortality in nematode-treated tests was significantly higher than control worker mortality for the two tested nematodes (treatment: $P = 0.005$, time: $P < 0.001$, and interaction: $P = 0.024$).

The nematode IBCB 24 caused higher *M. floricola* worker mortality in the first six days after nematode application. After this period, the two tested nematodes showed a similar mortality curve. The observed mortality curve on control

Table 3: Frequency of isolated fungi from ant nests and soil control samples.

	Frequency	Percentage
Isolated fungi from ant nests		
B eauveria bassiana only	3	4.8%
Metarhizium anisopliae only	6	9.7%
Metarhizium anisopliae +, *Beauveria bassiana*	1	1.6%
Paecilomyces sp. +, *Metarhizium anisopliae*	1	1.6%
Paecilomyces sp. + *Metarhizium anisopliae*	2	3.2%
Paecilomyces sp. only	9	14.5%
Nothing found	40	64.5%
Total	62	100%
Isolated fungi from soil control samples		
Beauveria bassiana only	6	9.5%
Beauveria bassiana + *Paecilomyces* sp.	1	1.6%
Beauveria bassiana +*Metarhizium anisopliae*	1	1.6%
Metarhizium anisopliae only	5	7.9%
Metarhizium anisopliae + *Paecilomyces* sp.	1	1.6%
Paecilomyces sp. *Beauveria bassiana* + *Metarhizium anisopliae*	1	1.6%
Paecilomyces sp. + *Beauveria bassiana*	1	1.6%
Paecilomyces sp., Metarhizium anisopliae	2	3.1%
Paecilomyces sp. + *Metarhizium anisopliae* + *Beauveria bassiana*	1	1.6%
Paecilomyces sp. only	12	19%
Nothing found	32	50.8%
Total	63	100%

Table 4: Isolation of *Beauveria bassiana, Metarhizium anisopliae,* and *Paecilomyces* sp. inside ant nests and in soil control samples.

Beauveria bassiana isolation		Control		Total
		No	Yes	
NEST	No	50 (82.0%)	7 (11.5%)	57 (93.4%)
	Yes	2 (3.3%)	2 (3.3%)	4 (6.6%)
Total		52 (85.3%)	9 (14.8%)	61 (100%)
Metarhizium anisopliae isolation		Control		Total
		No	Yes	
NEST	No	46 (75.4%)	5 (8.2%)	51 (83.6%)
	Yes	5 (8.2%)	5 (8.2%)	10 (16.4%)
Total		51 (83.6%)	10 (16.4%)	61 (100%)
Paecilomyces sp. isolation		Control		Total
		No	Yes	
NEST	No	40 (65.6%)	10 (16.4%)	50 (82.0%)
	Yes	2 (3.3%)	9 (14.8%)	11 (18.0%)
Total		42 (68.9%)	19 (31.2%)	61 (100%)

treatments showed progressive growth along the 33 days of evaluation due to stress caused by laboratory manipulation (Figure 1).

In this study, ant workers did not show any signs of infection by nematodes, but immatures were infected in the first days after application. We did not confirm the death of workers that received nematode applications, however, no nematode was observed in the traps.

Despite mortality of adult *M. floricola* workers throughout the test, no reduction of the colony was noticed in any treatment. There was no reduction in the amount of brood and queens. Throughout the observation period, all forms of immature individuals (eggs, larvae, and pupae) were observed in all colonies, but there was no production of alates. This observation is probably not correlated with the application of nematodes, since the control and the rearing colonies, which originated the ant population for the treatments, also did not show any males or ergatoid gynes.

The experimental colonies did not reduce in size, and sometimes a small increase of individuals was observed.

Grooming behavior was observed among workers that received nematode application. During the first evaluation days, the workers moved slower and constantly cleaned themselves. All treated groups showed an attempt to colony budding. Workers moved with the brood to the edges of the Petri dishes, leaving the center of the colony empty, probably due to a higher concentration of nematodes in this place.After attempted budding was observed, a new Petri dish was offered for all experimental colonies and they all moved to this new environment. Control colonies moved only half of the total brood, and workers, occupying both Petri dishes. Ant workers perceive the presence of nematodes and quickly move away healthy brood leaving only infected brood behind. There was not enough time for nematodes to infect ant individuals before they moved to other places free of nematodes.

After this period of evasion noticed in the first week of evaluation, the colonies showed no sign of infection. The ergatoid gynes continued oviposition and the colonies gradually increased in number of individuals. Dead workers remained distributed throughout the tray where colonies were kept and were not separated by live ant workers as usually happened with dead ants in the control colonies.

Behavioral responses, such as colony budding, show that the ants did suffer with nematode presence.

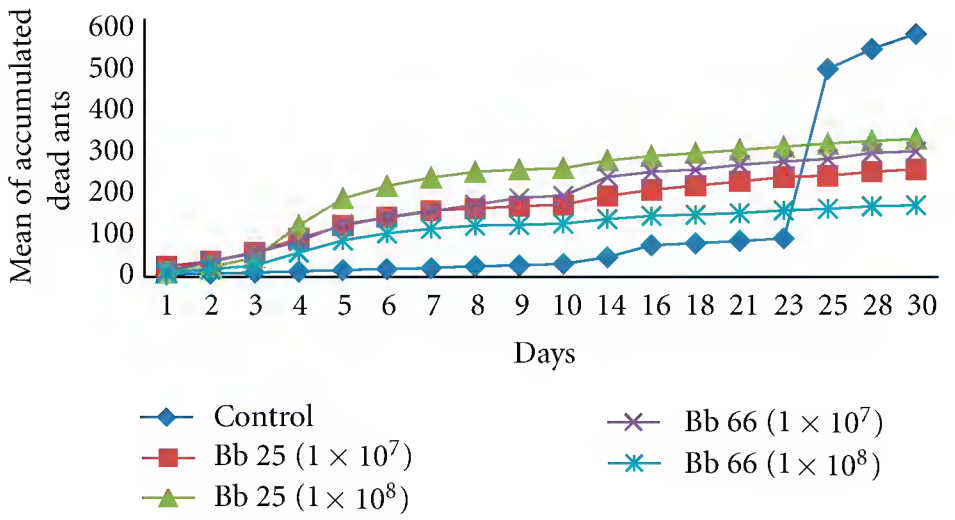
3.4.2. Susceptibility to Entomopathogenic Fungi. Both concentrations of *B. bassiana* isolated from ant nests (Bb 25 1×10^7 and Bb 25 1×10^8) and from the Entomopathogenic Collection (Bb 66 1×10^7 and Bb 66 1×10^8) caused higher *M. floricola* workers death in both tested concentrations (Figure 2). Ant mortality caused by the two *B. bassiana* isolates was significantly higher than the control and the other tested fungi (treatment: $P < 0.001$, time effect: $P < 0.001$, and interaction: $P < 0.001$) over the 30-day evaluation.

In the present study, mortality of *M. floricola* workers treated with *Paecilomyces* sp. P 405 (from the Entomopathogen Collection) was considerably high. Similar observation was not obtained from applications with *Paecilomyces* sp. isolated from ant nests. None of the *M. anisopliae* isolates in any concentrations killed significant numbers of workers in our experiment. None of the *M. anisopliae* isolates in any concentrations killed workers in our experiment (Figure 2).

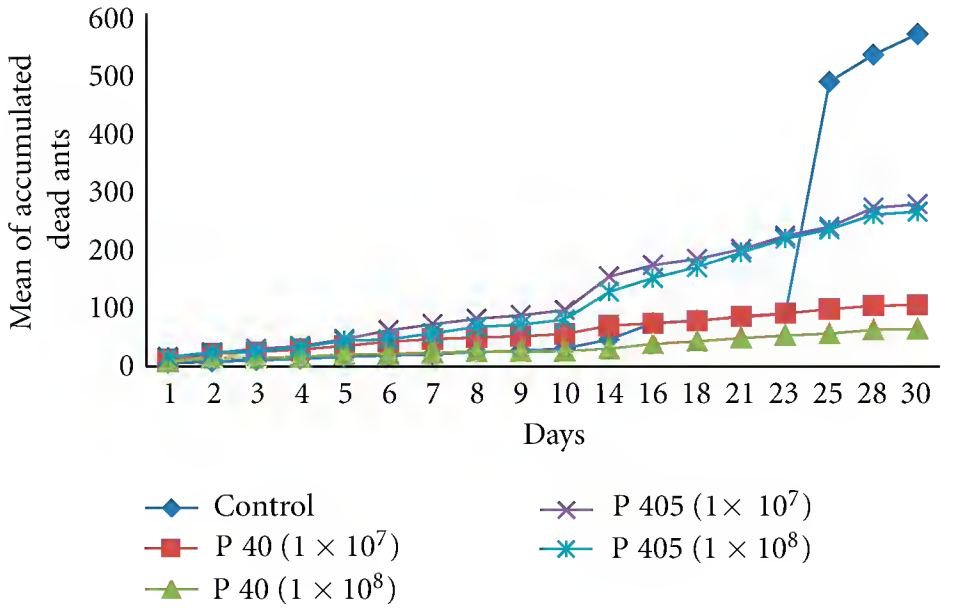
During the application of the fungal suspensions *M. floricola* workers were also crowded in the center of the Petri dishes. This behavior may have prevented large doses of fungal spores on a number of individuals, including the brood.

We observed that mortality of workers started to increase on the third day after fungal applications. Evaluation of fungal growth on the *M. floricola* worker cadavers also confirmed the high mortality due to fungi contamination from the third day. The highest confirmed mortality rates were obtained from the first to the tenth day after application. After this period the workers continued to die in greater numbers than those of the control colonies, but fungal growth on cadavers was no longer observed.

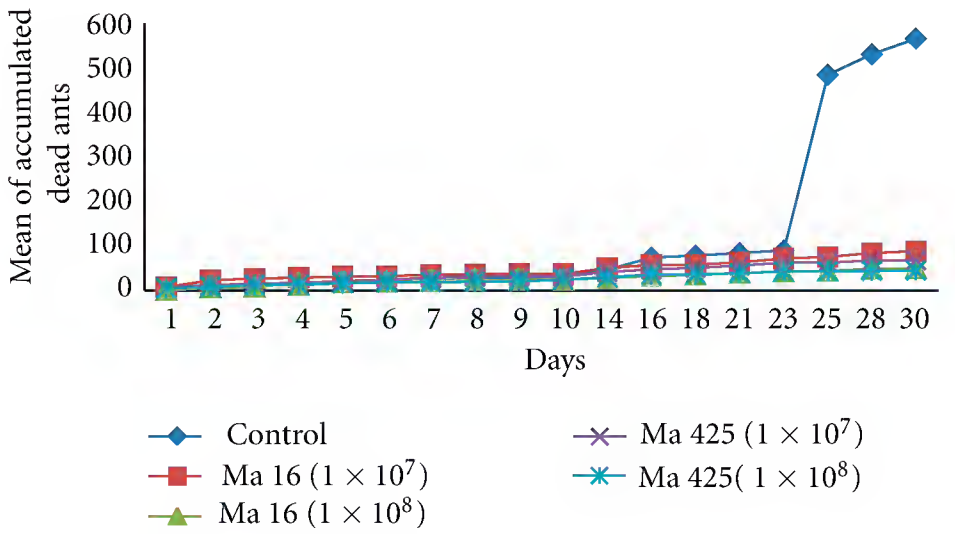
During the experiment, most colonies did not increase in size, but there was no apparent decrease. Treated workers constantly cleaned themselves and moved slowly, but there was no decrease in foraging. There was no decrease in the amount of brood and queens in any colony. All immature stages were present but we did not observe the presence of alates, including the control colonies.



(a)



(b)



(c)

FIGURE 2: Mean of cumulative mortality of *Monomorium floricola* workers, from five experimental colonies, submitted to the application of entomopathogenic fungi in different concentrations. Bb: *Beauveria bassiana*; P: *Paecilomyces* sp. Ma: *Metarhizium anisopliae*.

During the first days of evaluation, workers cleaned themselves probably trying to get rid of the fungal conidia. Unlike colonies treated with nematodes, no colony budding was observed when ants were treated with entomopathogenic fungi. Uninfected workers did not separate dead bodies maybe to avoid colony contamination.

4. Discussion

4.1. Entomopathogens Isolated from Invasive Ants and Tests of Their Pathogenicity. In this work entomopathogens were collected from *Solenopsis* spp. as other authors have already registered. Entomopathogens are commonly found on ant workers [16–18] and several of them have been collected in South American regions and proved as pathogenic to ants, especially *Solenopsis* [19, 20]. *B. bassiana* has been isolated from *Atta sexdens rubropilosa* and *Solenopsis* workers [21]. *M. anisopliae* and *B. bassiana* were isolated from *S. invicta* and *A. sexdens piriventris* [22, 23] in areas with no plant cover.

4.2. Susceptibility to Entomopathogenic Nematodes. The use of the nematodes *S. carpocapsae* and *Heterorhabditis* sp. for ant control has been successfully reported by several researchers [6, 24–27]. Poole [24] observed that *Solenopsis* sp. brood and alates were susceptible to a *S. carpocapsae* isolate when confined in small bottles containing infected soil. However, the adult workers were not much susceptible to their action. Drees et al. [6] also reported that nematodes caused mortality of *Solenopsis* sp. larvae, pupae, and alate, although the adult workers were not susceptible to the nematode. Ant workers vigorously groomed the brood, alates and themselves when the nematode was present. This defensive behavior probably removes the nematodes and was also observed by us.

As our experimental colonies did not reduce in size, and we could observe a small increase of individuals, it can be explained as an attempt by the colonies to recover from the control action. Zarzuela [28] also observed a recovery of the colonies of the same species in response to applications of chemical insecticides. If there is a disruption, whether chemical or biological, the ants tend to respond by increasing the number of individuals. Self-grooming and allogrooming, behaviors that we have observed, may impede or facilitate the spread of infection and disease according to Oi and Pereira [29]. While grooming among individuals of the colony may reduce infection by removal of the pathogen, it may also cause the spread of the disease, since the transfer of this pathogen can occur among individuals. The spread of pathogens as a result of grooming has not been observed in ants, but it is well documented on termites [30]. Drees et al. [6] reported that continuous brood, alate, or self-cleansing by workers of *Solenopsis* sp. after exposure of *Steinernema* and *Heterorhabditis* is an attempt to remove these nematodes.

Ant behavior changes caused by nematodes were also observed by other authors, besides grooming and colony recovery. Jouvenaz et al. [31] reported that *S. invicta* colonies displaced infected soil after treatment with *Steinernema* and *Heterorhabditis*. Drees and colleagues [6] observed that a significant number of ants abandoned their colonies when they received nematodes, forming new nests nearby. Budding after nematode treatment limits its use to control ant colonies in the field and we could observe such behavior in our experimental colonies.

4.3. Susceptibility to Entompathogenic Fungi. Many studies have reported the efficacy of *B. bassiana* in controlling ants. Alves and Gómez [21] observed high infectivity of this fungus on *Atta sexdens rubropilosa*. Pereira and colleagues [32] used the strain 447 of *B. bassiana* on workers of *S. invicta* and obtained 100% infection and mortality. Also for this same ant species, Stimac et al. [12] reported that almost 90% colonies treated with *B. bassiana* were killed. In 1995, Kelley-Tunis et al. [33] described a higher mortality of *Camponotus pennsylvanicus* workers when exposed to *B. bassiana* than when exposed to *M. anisopliae*.

In our study, *Paecilomyces* sp. P 405 killed a number of *M. floricola* workers but the same fungi isolated from ant nests were not efficient. Loureiro and Monteiro [34] applied *P. farinosus* in several concentrations on *A. sexdens sexdens* and reported 80% mortality of ant soldiers in the first four days after application.

Besides, *M. anisopliae* did not kill significant numbers of *M. floricola* ant workers, similar studies have demonstrated successful application of this fungus on ants [21, 23, 34–37]. Nevertheless, fungi are less effective when applied to colonies [35, 36], as occurred in this study. This decrease in efficacy has been attributed to a number of behaviors and chemical factors that ants exhibit [37].

A more effective alternative considered by several authors [37–41] is bait formulation in order to make the ants carry the fungus to the interior of the colonies. Although sometimes ants may perceive the presence of entomopathogens, these can be masked by the addition of attractive food [38, 39].

Formulations may play important role in fungal efficacy against ants. Silva and Diehl-Fleig [42] were successful when using spore suspensions of *M. anisopliae*. They recorded a decline of *A. sexdens piriventris* foraging for six days after application. However, direct application to the nests, is time consuming and impractical as a viable strategy in large areas [39, 42].

In the study conducted by Stimac and colleagues [12], the highest concentrations of *B. bassiana* conidia resulted in a higher mortality of *S. invicta*. The authors emphasize two important points in order to determine the optimal dose for higher ant mortality: application method and ant behavior. The authors observed that, when a large number of workers receive a fungal application, the ants tend to form a ball of bodies. It is difficult to reach all the individuals with the suspension of fungi. Due to cuticle composition, conidial suspensions are repelled by the mass of ants. Thus, the workers remaining in the center of the ant mass can not receive the same amount of treatment as the ants at the periphery of the ant mass.

We observed that mortality of workers started to increase on the third day after fungal applications. Increased mortality curves from the third day after entomopathogenic fungi application has been described since the initial studies of Stimac et al. [12, 19] and Alves et al. [20]. Such results are related to the necessary time for adhesion, germination, and growth of fungi before the insect death [43].

During the experiment, most colonies did not increase in size, but there was no apparent decrease. As with nematodes experiment, grooming behavior removes conidia or other fungal particles from the ant cuticle before infection occurs, and consequently reduces the infection rate of the entire colony. Observations under electron microscopy of *S. invicta* inoculated with *B. bassiana* conidia demonstrated the removal of conidia from the adult and larval integuments [29] and Sánchez-Peña and Thorvilson [44] inferred that self-grooming prevented ant infection by *Conidiobolus* conidia.

As nematodes, fungi can be promising alternatives for the control of urban ants. However, further studies should be conducted to determine the concentrations and application methods that can avoid the various defense behaviors of ants.

Acknowledgment

The authors thank the anonymous referees for their suggestions and corrections.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 281732, 18 pages
doi:10.1155/2012/281732

Research Article

Pollination Biology of *Potentilla recta* (Sulfur Cinquefoil) and Its Cooccurring Native Congener *Potentilla gracilis* in Northeastern Oregon

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Received 22 January 2012; Accepted 14 May 2012

Academic Editor: Tugrul Giray

Pollination biology of the invasive plant sulfur cinquefoil (*Potentilla recta* L.) and it's native cooccurring congener slender cinquefoil (*P. gracilis* Dougl. ex. Hook.) was studied from 2002–2004, at four sites in northeastern Oregon, USA The native cinquefoil flowered first for five weeks, followed by the invasive for five weeks, with two weeks overlap in mid-June. Invasive flowers attracted 74 species and 543 individuals; the native attracted 93 species and 619 individuals. The most important pollinators for the invasive, in order of importance, were: *Apis mellifera*, *Ceratina nanula*, *Halictus tripartitus*, *Lasioglossum sisymbrii*, and *Bombus rufocinctus*; for the native: *C. nanula*, *Trichodes ornatus*, *H. ligatus*, *L. sisymbrii*, and *L. olympiae*. The invasive produced higher numbers of seeds per plant, having greater mass per unit vegetation. Mean seed size was lower for the invasive when pollinators were allowed access to flowers, but seed size increased linearly with more complete exclusion of pollinators; the native showed no such response to pollinator exclusion. Compared to the native, nearly twice as many seeds germinated for sulfur cinquefoil (35.0% versus 19.5%), with seeds germinating over a longer period of time. Results are discussed as they relate to the invasiveness of sulfur cinquefoil relative to the native.

1. Introduction

Nonnative invasive plants are increasingly recognized as major threats to ecosystems worldwide, particularly in arid and semiarid regions [1]. In western North America, invasive plants have changed fire regimes [2], reduced livestock forage quality, damaged real estate and recreation values [3], and impacted biodiversity [2]. While their influence on biodiversity has been described well in terms of the structure of native plant communities, relatively less is known on their ecological relationships to other species, including those that play critical functional roles, such as pollinators.

Insects, especially bees, beetles, flies, and butterflies are known to pollinate a majority of vascular plant species worldwide; beetles alone have been observed to pollinate 211,935 species, or over 88% of the total species of vascular plants [4]. Insects also play a major role in crop reproduction: Williams [5] estimated that 84% of crop species in the European Union are pollinated by insects, and Buchmann and Nabhan [4] reported that 67 principal crop species are pollinated by insects worldwide, out of 84 listed (80%). The key to effective pollination service is diversity, since most native insect pollinator species visit only a small set of potential flowering plant species [6], and since many plant species are designed to be pollinated by only a small set of available pollinators [7]. As a result, most ecosystems require a diversity of both plants and pollinators in order for effective pollination to be carried out [8].

When exotic plants invade native communities, plant species diversity can decline, and this may lead to concomitant decreases in the diversity of native pollinator communities. Furthermore, the spread of invasive plants, especially those that reproduce only by seed [9], may be dependent on how successful they are at competing for the service of resident pollinators. Thus pollinators can act to exacerbate the spread of invasive plants, by providing a service that improves seed production and the colonization potential of these species [10, 11]. Unfortunately, basic

information on the pollination ecology of invasive plants is lacking for most species. This information is especially critical for those species that reproduce primarily by seed, particularly if seed viability depends on outcrossing [11].

This study describes the pollination biology of the invasive plant sulfur cinquefoil (*Potentilla recta* L; Rosaceae) and its native cooccurring congener slender cinquefoil (*P. gracilis* Dougl. ex. Hook.), in northeastern Oregon. Sulfur cinquefoil is native to Eurasia and was introduced into North America before 1900 [12]. It is now naturalized across much of the United States and southern Canada, occurring from British Columbia east to Newfoundland and Nova Scotia, south to Florida, and west to eastern Texas [12–15].

In northeastern Oregon, sulfur cinquefoil occurs in open grasslands, shrubby areas, and disturbed areas including old fields, roadsides, pastures, and fencerows [16]. Sulfur cinquefoil can be highly competitive and has been observed to invade bluebunch wheatgrass (*Pseudoroegneria spicata*) rangeland in good condition and to displace other invasive species at some sites [17]. Sulfur cinquefoil is unpalatable to most livestock and wildlife, primarily because of its high-tannin content [12, 18, 19]. In fact, cattle will selectively graze spotted knapweed, another unpalatable species, in preference to sulfur cinquefoil [19]. As a consequence, overgrazing, which reduces competition from grass and other competing vegetation, generally favors sulfur cinquefoil [20].

Like its native congeners [21], sulfur cinquefoil is a long-lived perennial forb, having one to several erect, stout stems 30–70 cm tall growing from a woody caudex [12, 17, 18]. Peak flowering generally occurs in late June, depending on locality [12, 18]. Sulfur cinquefoil reproduces primarily by seed, and although self-fertilization can occur, most seeds are produced by cross-fertilization [22]. Seeds do not have a special dispersal mechanism [22]. Seeds germinate naturally at anytime during the growing season [23], and most vegetative growth occurs early the following spring [19].

In northeastern Oregon, sulfur cinquefoil cooccurs with its native congener *P. graclis* at many localities [24], and this presented the opportunity to study its pollination biology relative to the native. In particular, a comparative study of plants of both species living side by side could shed light on the extent to which the invasive has evolved distinct strategies to attract and retain pollinators, relative to the native congener. The current study compares the pollination biology of *P. recta* and *P. gracilis* by investigating respective flowering phenology, pollinator community structure, pollinator preference, nectar rewards, fidelity of pollen transfer, and influence of pollinator exclusion on seed set, seed size and number, and germination timing and rate.

2. Study Sites and Methods

The study was conducted between May 2002 and July 2004, in northeastern Oregon, where cinquefoil grows in small meadows intermixed with trees and shrubs (Figure 1). The general area experiences a Pacific Maritime Climate, warm and dry from late June to October, and cool and wet from November through May. Between 1965 and 2005, annual mean daily high temperature in La Grande, OR was 16°C, annual mean low temperature was 3°, and annual precipitation was 43.5 cm. Four study sites were selected for this study (Figure 1): the "Foothill" site (800 m elevation), just south of and closest to the largest municipality (La Grande OR, USA), was also the most dominated by sulfur cinquefoil (>95% *P. recta*); the "Rice" site was at slightly higher elevation (1000 m) on Glass Hill Road, 5 km southwest of La Grande, and here *P. recta* represented about 70% of total *Potentilla* cover; the "Ham" site (elevation 900 m) was located on Hamburger Hill, between Imbler and Elgin, 15 km northeast of La Grande, at which *P. recta* represented 50% of total *Potentilla* cover and the "Morgan" site was located at Morgan Lake, 10 km west of La Grande (1200 m elevation), and here *P. recta* represented just 10% of the total *Potentilla* cover.

In May 2002, we established five circular 400 m^2 plots (11 m radius) at each of the four study sites, within which most subsequent fieldwork was undertaken. Plots were selected so as to represent the approximate invasive to native composition of *Potentilla* species at that site. To determine flower phenology, relative flower availability was assessed at weekly intervals throughout each flowering season at each site, by counting the number of open flowers of each species (invasive or native) within each 400 m^2 plot. To determine the structure of the pollinator community of each cinquefoil species through time (species composition and relative abundance), we collected and identified all flower visitors within each plot at weekly intervals throughout each flowering season (2002, 2003, and 2004). By combining data on pollinator community structure for the two plant species within each plot with the relative abundance of flowers for each species, we could determine pollinator preference, by calculating an "electivity" index [25] for each flowering species, with the use of the following equation:

$$\mathrm{EI}_a = \frac{R_a - P_a}{R_a + P_a}, \tag{1}$$

where EI_a is Electivity index for plant species a, R_a is proportion of total pollinator population visiting plant species a, and P_a is proportion of plant species a in total flowering population.

This index will range between (−1), indicating total avoidance by pollinators of that plant species, to nearly (+1), which would indicate total dominance by that plant species of the pollinator resource. A value of (0) would indicate no preference for flowers of the given species of plant.

To gain insight into the potential efficacy of insects for distributing cinquefoil pollen, we removed and identified pollen from at least ten individuals of the 20 most common flower visitor species. Pollen was brushed off the bodies of randomly selected pinned individuals onto glass slides and preserved with standard methods. Pollen was identified with the use of a reference collection obtained by extracting pollen from flowers curated in the plant collection at Eastern Oregon University. In June and July 2003, we measured nectar quality in flowers of *P. recta* and *P. gracilis*, using a hand-held refractometer. Flowers of both species were collected, centrifuged, and a capillary tube was used to extract and measure the quality of nectar (% solute).

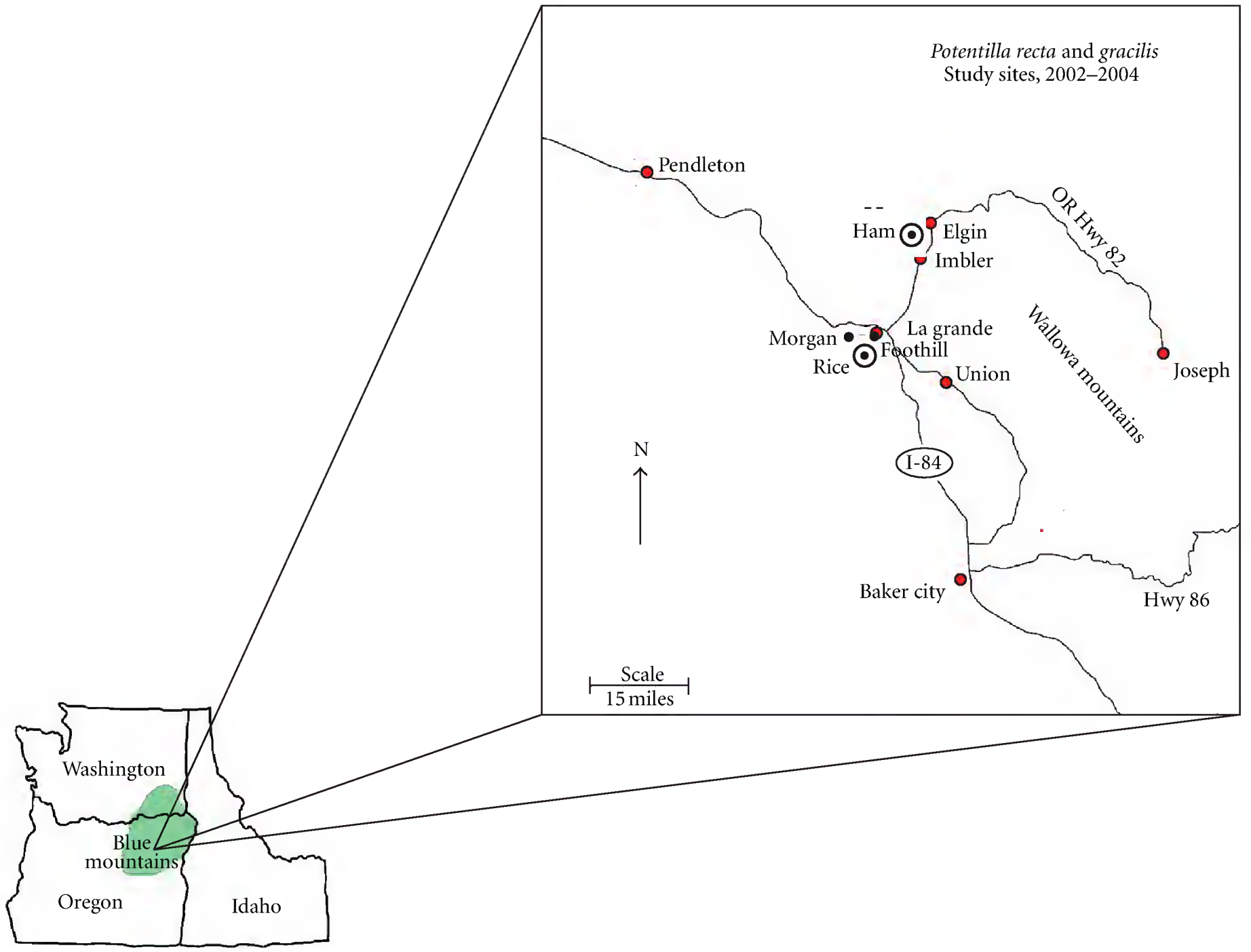

FIGURE 1: Map of *Potentilla* study sites, northeastern Oregon, 2002–2004.

Between late May and early July 2003, at two of the four study sites (Ham and Rice), we conducted a pollinator exclusion experiment designed to measure the potential influence of pollinators on seed set, seed size, and germination rate. Following the general protocol of Barthell et al. [11], five treatments were applied, four of which featured flower-head exclusion bags that varied in mesh size, designed to exclude pollinators of various sizes (Figure 2): (1) 1-mm mesh size: excluded all pollinators, regardless of size; (2) 3-mm mesh size: allowed access to the smallest pollinators, such as most Halictids and small Syrphid flies, but excluded medium and large pollinators such as most Apids, Megachilids, Andrenids, and large Syprhid and Bombyliid flies; (3) 5-mm mesh size: allowed access to small and medium-sized pollinators, but excluded the largest pollinators such as Bombids; (4) 10-mm mesh size: a "sham" cage, designed to test for bag effects *per se*: technically allowed access to all pollinators, regardless of size; and (5) no bag: flowerheads were left in the natural state, which allowed uninhibited access to all pollinators. One complete block of the five treatments was applied to a total of 240 flowerheads, 120 at each site, with each flowerhead representing an individual cinquefoil plant. At each site, we established four separate transects, separated by at least 100 m, along which we positioned 30 randomly selected plants, 15 of which were sulfur cinquefoil, alternating with 15 that were native cinquefoil. Bags were installed at least one week prior to flowering (late May to early June), and because flowerheads continued to expand during the experiment, bags had to be regularly re-positioned to accommodate new growth. Throughout the experiment, we visited bag installations on a weekly basis, to check for bag damage or other problems in installation, to record unexpected ingress of insects into the bags, and to count visitors on unbagged flowers of each species. Once flowering ceased, experimental bags were replaced with opaque cotton "seed bags," to insure that no seeds escaped from flowerheads as the seeds within them matured in the weeks following the cessation of flowering. After flowerheads had stopped growing and had clearly senesced (by the end of July), flowerheads were removed from plants and taken back to the lab for processing. In early August, flowerheads were oven-dried, dissected, and all seeds removed, counted, and weighed. To check for potential effects of seed predation on germination, a

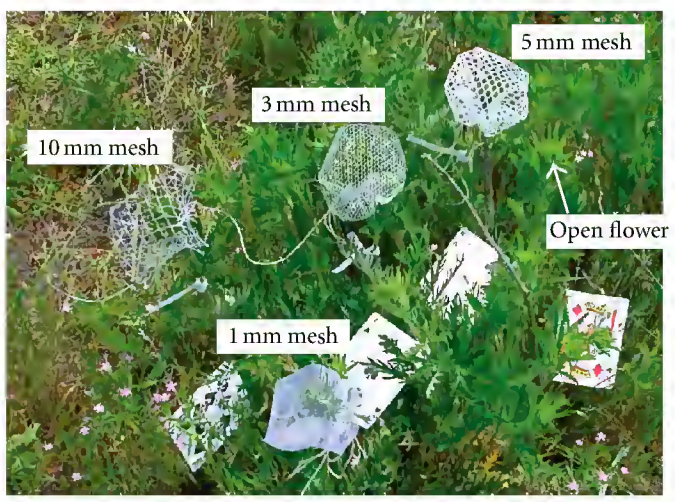

Figure 2: Photograph of a block of the five treatments deployed in the cinquefoil pollinator exclusion experiment: open flower (all access); 10 mm mesh size (sham cage, all access); 5 mm mesh (excludes large pollinators); 3 mm mesh (excludes large and medium pollinators); 1 mm mesh (excludes large, medium, and small pollinators).

total of 30 flowerheads of each species, 15 from both Rice and Ham sites, were dissected and checked for evidence of seed predation. To determine germination success over time, a subset of the total seeds (typically > 30) within each flowerhead were randomly selected, placed on moist filter paper within a covered petri dish, and monitored weekly for one year, and cumulative germination was determined.

Data on community structure are presented descriptively for all four study sites, as lists of species found through each of the three sampling seasons (2002, 2003, and 2004). The 20 most commonly collected pollinator species for each plant species are then compared descriptively. The ordination method "Nonmetric multi-dimension scaling" (NMS) [26] was used to characterize sites based on their composition and relative abundance of species, and then axes are correlated with site factors in an attempt to explain among-site and between-species patterns of community structure. NMS is ideal for ordination of most community data, because the technique is nonparametric, and thus does not assume any underlying distributional properties in the data set. Data on flower preference, using the electivity index were used to augment insights on the nature of patterns of pollinator use of the two plant species. For the bag experiment, we analyzed for the fixed effect of bag type on seed size, seed number per head, seed mass per head, and germination timing and rate with the use of a mixed general linear model, that included plant species, transect, and site as random factors.

3. Results

A total of 1,045 individual flower visitors were collected at the four sites over the three-year study period, comprising four orders, 36 families, and 111 species of insects (Table 7). Sulfur cinquefoil flowers attracted 74 species and 543 individuals, 16% of which were European honeybees (*A. mellifera* L.), while the native cinquefoil attracted a more diverse fauna of 93 species and 619 individuals, only 2% of which were honeybees. The 20 most commonly collected flower visitor species represented nearly 69% of the total individuals collected for each cinquefoil species (Table 1). Most pollinator species were "rare", reflected by the fact that 50 of 93 insect species observed on the native (53%) were represented by one or two individuals; for sulfur, 41 of 74 species were so represented (54%). Based on a combination of abundance and the presence of pollen on their bodies, the five most important pollinators for sulfur cinquefoil, in order of importance, were likely to be *A. mellifera, Ceratina nanula, Halictus tripartitus, Lasioglossum sisymbrii*, and *Bombus rufocinctus*; for the native, the most important pollinators were likely to be *C. nanula, Trichodes ornatus, H. ligatus, L. sisymbrii*, and *L. olympiae*. None of the 10 principle pollinators of each species were abundant throughout each respective flowering season, although for the native cinquefoil, most species were present throughout June, and for sulfur cinquefoil, most species were present from mid-June to mid-July (Table 2).

Although the pollinator fauna of the native cinquefoil was more abundant and rich than the fauna of sulfur cinquefoil, temporal (among-year) variance to mean ratios for pollinator abundance and richness for the native were roughly fourfold higher than for sulfur, and the spatial (among-site) variance to mean ratios were more than tenfold higher for the native, compared to sulfur (Table 3). Thus, it was much easier to predict both counts and species richness at any give time and place for sulfur cinquefoil, compared to the native. For example, despite equivalent sampling efforts at all sites each year, the native cinquefoil had very low abundance and richness of pollinators at the Foothill site, where sulfur cinquefoil dominated in percent cover (>90% sulfur), but very high abundance and richness of pollinators at the Morgan site, where the native dominated (90% native). In addition, the native pollinator fauna was roughly twice as abundant and three times as rich in 2003, as it was in the other two years (2002 and 2004). As a consequence, the invasive sulfur cinquefoil had a much more constant community of flower visitors over space and time compared to the native.

NMS ordination demonstrated few clear patterns of among-site, or between-cinquefoil species differences in pollinator communities. The most apparent pattern was the significant difference in community structure among survey years (Figure 3). The distinctiveness of the fauna in 2002 was represented best by Axis 2, with *C. nanula* (Apidae) and *L. sisymbrii* (Halictidae) having the highest correlations with Axis 2. The species that most indicated the position of the 2004 site samples, also correlated closely with Axis 2, were *Panurginus* sp. (Andrenidae) and *Coenonympha tullia* (Satyridae), followed by *Eristalis hirta* (Syrphidae), *Hylaeus episcopalis* (Hylaeidae), and *H. ligatus* (Halictidae). Axis 1 best separated 2003 as a distinctive year, and its strongest indicators were *E. hirta*, *L. olympiae*, *C. acantha*, and *H. farinosus* (Table 1).

Under field conditions of equal flower dominance, we were able to acquire preference data for nine taxa of pollinators (Table 4). Of these, only the European honeybee and two *Megachile* speices exhibited preference for sulfur cinquefoil (electivity index > 0), while two bee genera (five species) showed no preference (*Halictus, Bombus*), and 11 species in

Table 1: List of most commonly observed pollinator species (total abundance ≥ 5) for sulfur and native cinquefoils, ordered by abundance for each species, at four study sites in northeastern Oregon, 2002, 2003, and 2004. KEY to abbreviations: MO: Morgan Lake; FH: Foothill; RI: Rice; HH: Ham; NAT: Native Cinquefoil; SULF: Sulfur Cinquefoil.

Pollinator species	2002	2003	2004	MO	FH	RI	HH	TOT NAT
Panurginus sp. (UID)	0	27	68	11	0	15	69	95
Eristalis hirta	0	32	9	21	9	8	3	41
Ceratina nanula	40	1	0	0	0	2	39	41
Trichodes ornatus	16	6	10	2	0	23	7	32
Lasioglossum sp. (UID)	3	6	17	5	0	15	6	26
Lasioglossum olympiae	0	21	0	7	0	2	12	21
Halictus ligatus	7	8	5	5	0	1	14	20
Lasioglossum sisymbrii	17	0	0	3	0	2	12	17
Hylaeus episcopalis	0	11	4	7	0	7	1	15
Apis mellifera	0	11	3	1	5	0	8	14
Andrena sp. (UID)	0	11	3	8	1	5	0	14
Coenonympha tullia	0	5	8	1	0	5	7	13
Halictus tripartitus	1	11	0	4	0	1	7	12
Evylaeus sp. (UID)	0	11	0	9	0	0	2	11
Halictus sp. (UID)	1	7	2	0	0	2	8	10
Ceratina sp. (UID)	0	0	10	0	0	0	1	10
Colias sp. (UID)	7	0	0	0	0	0	7	7
Ceratina acantha	0	7	0	2	0	2	3	7
Bombus rufocinctus	0	7	0	3	1	2	1	7
Speyeria sp. (UID)	6	0	0	0	0	3	3	6
Osmia sp. (UID)	0	2	4	2	0	2	2	6
Halictus farinosus	1	5	0	2	2	2	0	6
Epicauta puncticolis	0	3	2	1	0	3	1	5
Total abundance	144	299	176	153	25	164	268	619
Total richness	48	133	55	56	13	51	54	93
Pollinator species	2002	2003	2004	MO	FH	RI	HH	TOT SULF
Apis mellifera	41	28	16	0	42	3	4	85
Lasioglossum sp. (UID)	19	1	18	4	6	2	8	38
Eristalis hirta	0	13	17	12	1	6	2	30
Ceratina nanula	24	1	0	5	8	3	9	25
Halictus tripartitus	15	5	5	9	5	7	4	25
Hylaeus episcopalis	0	9	12	9	0	7	5	21
Bombus bifarius	8	10	1	5	5	5	4	19
Lasioglossum sisymbrii	17	2	0	0	4	8	7	19
Ceratina sp. (UID)	0	7	10	9	0	0	8	17
Bombus rufocinctus	4	5	5	4	1	8	1	14
Halictus ligatus	1	1	12	1	7	3	3	14
Andrena prunorum	5	4	2	2	2	5	2	11
Panurginus sp. (UID)	0	2	8	4	5	0	1	10
Halictus sp. (UID)	1	0	8	0	6	1	2	9
Andrena sp. (UID)	0	3	5	4	0	4	0	8
Trichodes ornatus	6	0	1	1	0	5	1	7
Lasioglossum titusi	4	3	0	1	1	3	2	7
Megachile perihirta	2	2	3	2	1	2	2	7
Total abundance	222	164	157	107	144	127	102	543
Total richness	63	88	70	40	30	44	41	74

Table 2: Phenology of ten most commonly observed flower visitors of *Potentilla gracilis* (native) and *P. recta* (exotic), at four sites in Northeastern Oregon, 2002–2004.

Pollinator species	May 16–31	June 1–15	June 16–30	July 1–15	July 16–31
			P. gracilis		
Coenonympha tullia	46.2	38.5	15.4		
Trichodes ornatus	12.5	37.5	40.6	9.4	
Panurginus sp.	10.5	62.1	25.3	2.1	
Halictus ligatus	4.8	42.9	52.4		
Ceratina nanula		97.6		2.4	
Lasioglossum olympiae		85.7	14.3		
Lasioglossum sisymbrii		76.5	23.5		
Eristalis hirta		70.7	14.6	14.6	
Apis melifera		50.0	42.9	7.1	
Hylaeus episcopalis		6.7	66.7	26.7	
			P. recta		
Halictus ligatus	42.9	21.4	28.6	7.1	
Bombus rufocinctus	7.1		92.9		
Bombus bifarius	5.3	47.4	47.4		
Eristalis hirta	3.3	76.7	20.0		
Apis melifera	1.2	64.7	32.9	1.2	
Lasioglossum sisymbrii		42.1	57.9		
Hylaeus episcopalis		38.1	61.9		
Andrena pronorum		36.4	63.6		
Halictus tripartitus		12.0	64.0	24.0	
Ceratina nanula			72.0	28.0	

Table 3: Summary data for pollinator surveys for sulfur and native cinquefoil at four sites in northeastern Oregon, 2002, 2003, and 2004.

Sulfur cinquefoil			Native cinquefoil		
Year	Mean abundance per site	Mean richness per site	Year	Mean abundance per site	Mean richness per site
2002	55.5	15.8	2002	36.0	12.0
2003	41.0	22.0	2003	74.8	33.3
2004	39.3	17.5	2004	44.0	13.8
Mean/Year	45.3	18.4	Mean/Year	51.6	19.7
Var	79.6	10.4	Var	418.5	139.1
Var/Mean	1.8	0.6	Var/Mean	8.1	7.1
Site	Mean abundance per year	Mean richness per year	Site	Mean abundance per year	Mean richness per year
Morgan	107	40	Morgan	153	56
Foothill	144	30	Foothill	25	13
Rice	127	44	Rice	164	51
Ham	102	41	Ham	268	54
Total	543	74	Total	619	93
Mean/Site	120.0	38.8	Mean/Site	152.5	43.5
Var	372.7	36.9	Var	9909.7	417.7
Var/Mean	3.1	1.0	Var/Mean	65.0	9.6

5 taxonomic groups demonstrated preference for the native (*C. nanula*; *Andrena*—2 spp; *Hylaeus*—2 spp, Syrphidae—3 spp, Coleoptera—3 spp). These data roughly correspond to survey data, when pollinator species are ordered in terms of relative abundance for each of the cinquefoil species (Table 1).

Estimates of percent sugar concentration in nectar were more than six-fold higher for sulfur cinquefoil than for

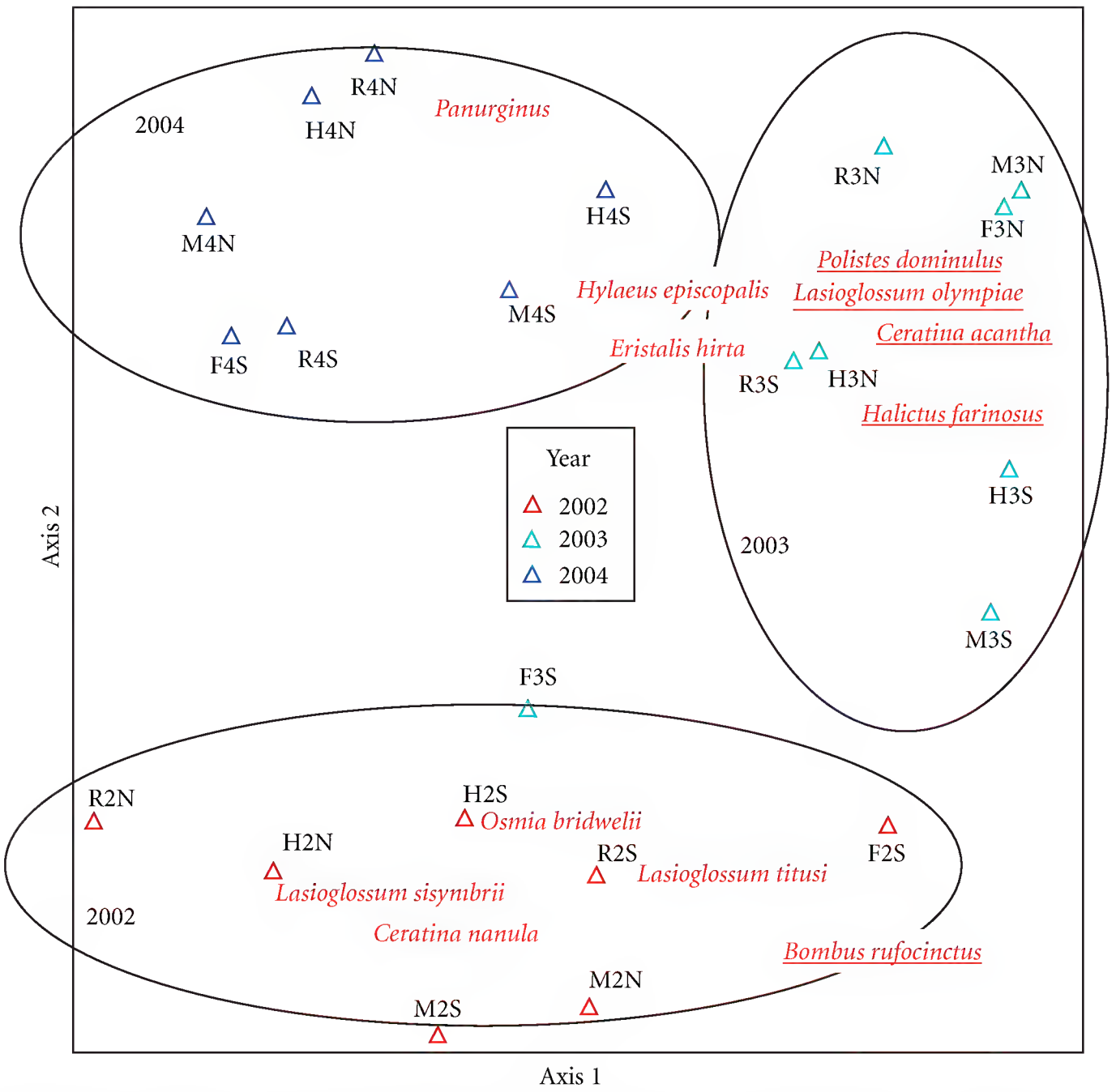

Figure 3: Ordination of sites by species for sulfur and slender cinquefoil pollinator communities at four sites in northeastern Oregon, 2002–2004. Key to sample acronyms (upright font): M = Morgan; F = Foothill; R = Rice; H = Ham; 2 = 2002; 3 = 2003; 4 = 2004; S = Sulfur; N = Native. Indicator species indicated in italicized font (Axis 2 correlates) and underlined font (Axis 1 correlates).

Table 4: Electivity indices for pollinator taxa under conditions of equal flower abundance. R: proportion of total pollinator population visiting plant sulfur cinquefoil; P: proportion of sulfur flowers among all cinquefoil flowers; E: electivity index = $(R_{\text{sulfur}} - P_{\text{sulfur}})/(R_{\text{sulfur}} + P_{\text{sulfur}})$.

Pollinator	Tot obs	No. of sulfur	No. of native	R	P	E
Apis mellifera	11	10	1	0.91	0.5	**0.29**
Megachile—2 spp.	5	4	1	0.80	0.5	**0.23**
Bombus—3 spp.	7	4	3	0.57	0.5	0.07
Halictus—3 spp.	21	9	12	0.43	0.5	−0.08
Andrena—2 spp.	13	3	10	0.23	0.5	**−0.37**
Ceratina nanula	19	4	15	0.21	0.5	**−0.41**
Hylaeus—2 spp.	5	1	4	0.20	0.5	**−0.43**
Syrphidae—3 spp.	6	1	5	0.17	0.5	**−0.50**
Coleoptera—3 spp.	10	0	10	0.00	0.5	**−1.00**

the native cinquefoil (59.0 ± 0.8 S.E. versus 9.6 ± 0.3 S.E.). These estimates correspond to observations indicating that honeybees were much more attracted to invasive flowers compared to the native.

When seed parameters are compared between the two species for the unmanipulated (open) treatment, several differences were observed. First, mean individual seed mass was significantly higher for the native compared to sulfur cinquefoil (0.207 mg mean seed mass ± 0.003 S.E. for the native, versus 0.172 mg ± 0.003 S.E. for sulfur), and these differences were consistent for both the Ham and Rice sites. Second, the native cinquefoil produced significantly fewer seeds per head than did sulfur (1202 seeds per head ± 103 S.E. for the native versus 1817 ± 104 S.E. for sulfur), although seed production by the native was significantly lower at the Rice site. Despite having significantly smaller

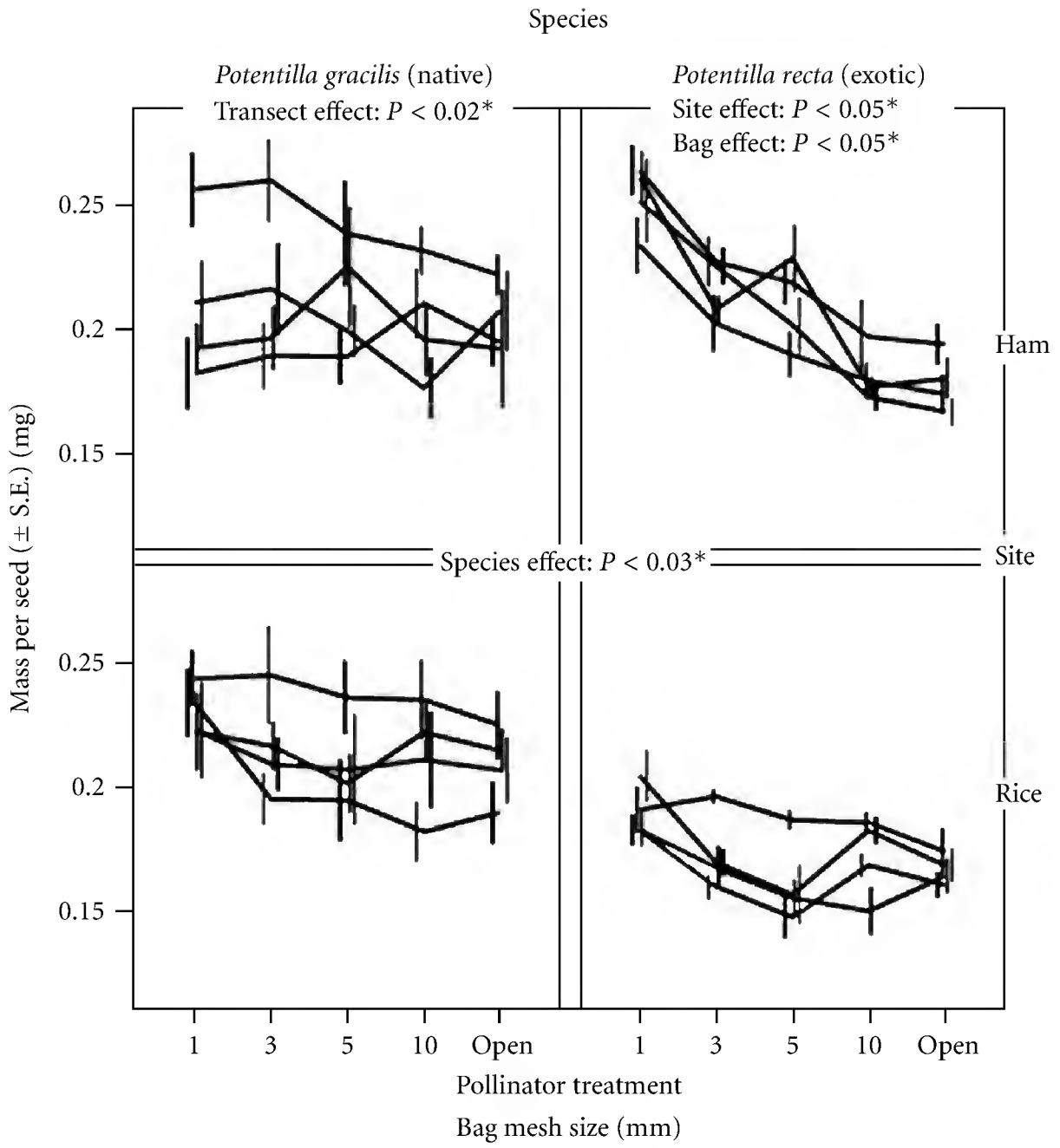

FIGURE 4: Mass per individual seed (mg) for *Potentilla gracilis* and *P. recta* seeds produced by flower heads exclosed by bags having mesh sizes designed to exclude various sizes of pollinators, along four transects at both Ham and Rice sites, northeastern Oregon, June-July 2003.

seeds, overall differences in seed number per head translated into significantly higher total seed mass per head for sulfur, compare to the native (0.31 g mass per head ± 0.02 S.E. for sulfur, versus 0.23 g ± 0.02 S.E. for the native). Once again however, native production was lower at the Rice site.

Pollinator exclusion at the Rice and Ham sites caused significant changes in seed parameters for both species of cinquefoils, but effects were much more pronounced for sulfur cinquefoil and were generally of greater magnitude at the Ham site. At both sites, sulfur cinquefoil plants produced progressively larger seeds as bag treatments became limiting to progressively smaller pollinators (Figure 4). This effect; however, was somewhat site-specific, with the Ham site exhibiting a more pronounced effect ($P < 0.0001$), compared to the Rice site ($P < 0.05$). This is reflected by the magnitude of increases in mean seed mass for sulfur cinquefoil between the open treatment and the most exclusive 1 mm bag treatment: at the Rice site, mean seed mass increased just 14% from 0.17 mg (± 0.003 S.E.) to 0.19 mg (± 0.004 S.E.), while at the Ham site, mean seed mass increased 41% from a mean of 0.18 mg (± 0.004 S.E.) to a mean of 0.25 mg (± 0.006 S.E.). For the native, mean seed mass did not generally increase with progressive decreases in bag mesh size, though at the Rice site, mean size mass increased slightly from 0.21 mg (± 0.004 S.E.) to 0.23 mg (± 0.008 S.E.). For the number of seeds per exclosed head, significant effects were observed only at the Ham site. For the native, although there was no significant bag effect overall for seed number, plants at the Ham site that had received the most exclusive bag treatment (1 mm) produced seed heads having significantly fewer seeds compared to the open treatment (928 seeds/head ± 85 S.E. versus 1538 ± 161 S.E.). For sulfur, the experimental results at the Ham site were also distinctively different than for the Rice site in terms of seed number per head. In particular, flower heads that were exclosed by the 1 mm bag produced only 1/2 of the total seeds per head compared to the open treatment (949 seeds/head ± 61 S.E. versus 1892 seeds/head ± 179 S.E.). In contrast, at the Rice site, plants of neither species responded significantly to treatments in terms of seed number per head. Overall however, for sulfur cinquefoil, the larger seeds observed in the 1 mm bag were produced at the expense of a significantly lower seed number per head, although this effect was much more pronounced at the Ham site. For total seed mass per head, effects were not obviously progressive when comparing all bag treatments, and also tended to vary with site, in much the same way as for the number of seeds per head. For the native, while there were no significant treatment effects on seed mass per head when all bag treatments were analyzed together, seed mass at the Ham site decreased significantly ($P < 0.01$) from 0.28 g per head (± 0.02 S.E.) in the open treatment to 0.18 g per head

(± 0.01 S.E.) for the 1 mm bag, representing a 36% decrease. At the Rice site; however, the decrease was only 9% and was not significant. For sulfur cinquefoil, once again seed mass per head decreased significantly only at the Ham site, from 0.33 g (± 0.03 S.E.) for the open treatment to 0.24 g (± 0.01 S.E.) for the 1 mm bag treatment, representing a 27% decrease; at the Rice site, both treatments produced a mean seed mass per head of 0.29 g. Finally, note that the among-site variation presented above was augmented by within-site variation, as reflected by the four transects located at each experimental site (Figure 4). In particular, note that variation among transects was substantial, both in mean seed mass, and in the pattern of response across treatments, especially for the native cinquefoil. Clearly, while pollinator exclusion had clear effects in some cases, the magnitude of spatial variation at two scales makes it risky to predict what might happen with a similar experiment at other sites.

There is no obvious explanation for the observed differences in treatment effects between the Rice and Ham sites. These two sites were similar in elevation, aspect, and general landscape conditions, and while seed productivity was much higher at Ham for the native, sulfur cinquefoil plants produced roughly similar seed numbers and seed mass at the two sites in the open condition. To assess whether the greater magnitude of effects at the Ham site could have been due to higher numbers of pollinators or a more diverse pollinator community there, we observed patterns of flower visitation during the experiment. These data indicate that site differences cannot be explained by either the number or community structure (Table 5) of pollinators that may have been excluded: the richness and species composition of pollinators observed at flowers of plants neighboring those that had received treatments were roughly similar for the Rice and Ham sites (Table 5), and there were actually more pollinators available at the Rice site compared to the Ham site, during the experiment. Moreover, if the more subtle effects of treatment at the Rice site was due to a lower level of pollinator service, we would expect that seed numbers and mass per head would be equally high for the open versus 1 mm bags, instead of equally low, as we observed. For example, at the Rice site, mean seed mass per head for the native in the open treatment was only 0.16 g (± 0.02 S.E.), compared to 0.28 g (± 0.02 S.E.) in the open condition at the Ham site. If differences in pollinator community structure or overall abundance were responsible for the lack of effect at Rice, then we would have expected both the open and 1 mm treatment to have seed mass equally high, and more similar to the Ham site. Clearly, some other factor or set of factors was responsible for the difference in treatment effects between the Rice and Ham sites.

When other aspects of seed biology for the two plant species was compared, there were three distinct differences observed. First, the proportion of buds within which evidence of seed predation was observed was 0.22 for the native, compared to just 0.01 for the invasive (Table 6). Second, the invasive *P. recta* invested proportionally greater resources in seed production compared to the native cinquefoil, with total seed mass per head three times that observed for the native (Figure 5). Third, less than 20% of native cinquefoil

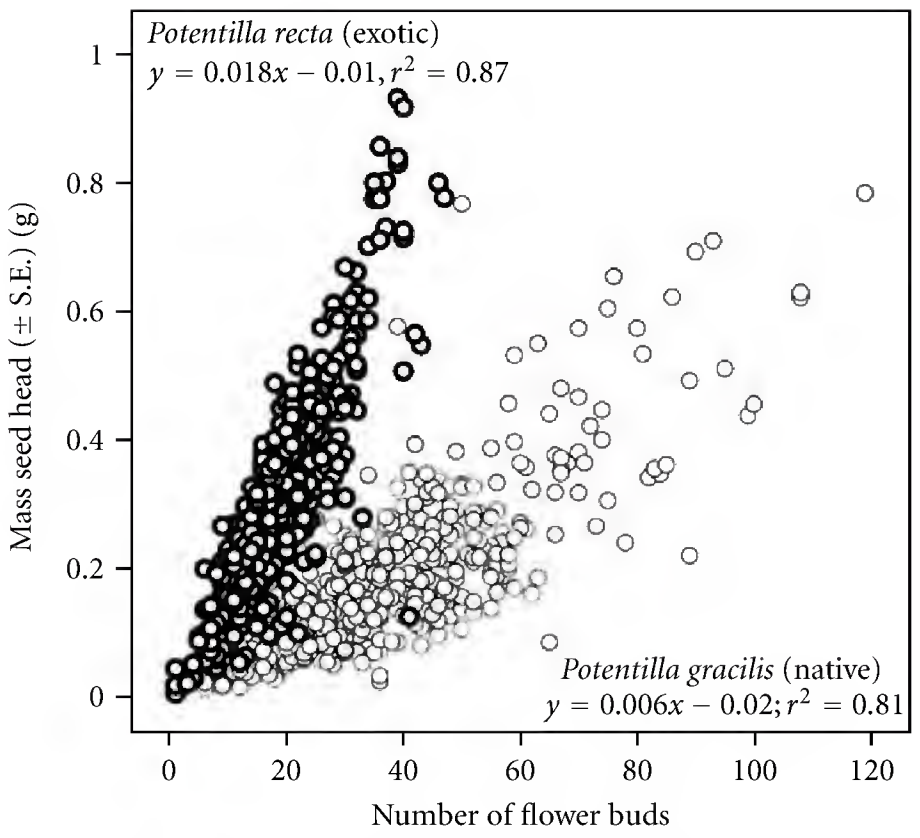

FIGURE 5: Mass of seed head (grams) as function of the number of buds in a head for *Potentilla gracilis* (native) and *P. recta* (invasive), at Rice and Ham sites, northeastern Oregon, June-July, 2003.

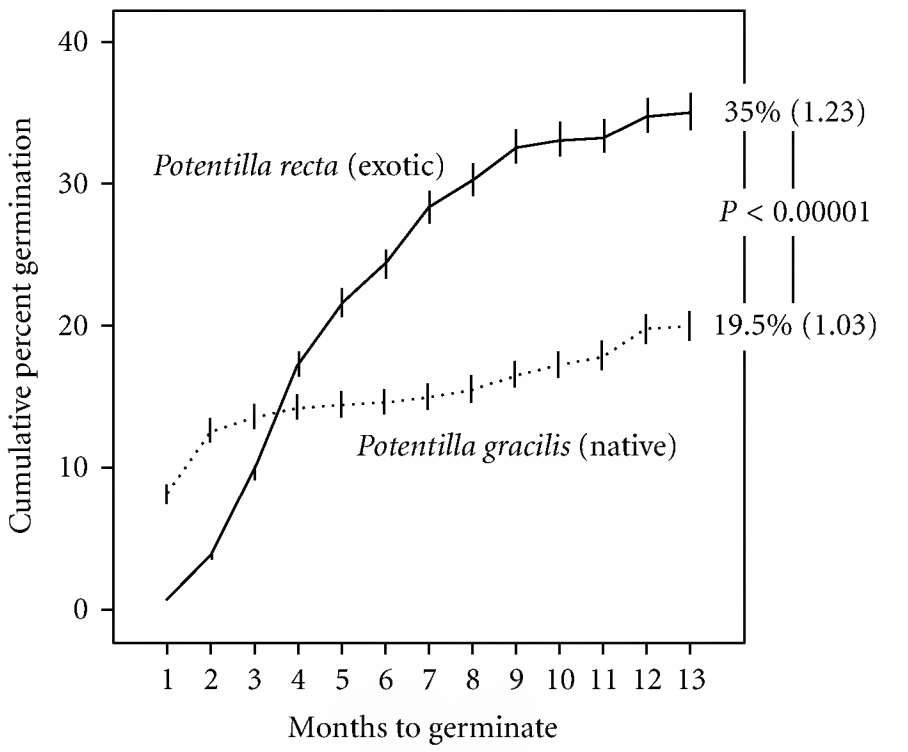

FIGURE 6: Cumulative percent germination by month for *Potentilla gracilis* (native) and *P. recta* (invasive), from seeds collected from plants at Ham and Rice sites, northeastern OR, July 2003–June 2004.

seeds germinated, and most germination occurred within two months after wetting, while 35% of sulfur cinquefoil seeds germinated, with germination occurring consistently for more than eight months after wetting (Figure 6). Finally, none of these germination parameters were significantly influenced by the pollinator exclusion treatment.

4. Discussion

Collectively, the flowers of cinquefoil attracted 111 insect species at four sites in northeast Oregon, but just 26 insect species comprised roughly 70% of total flower visitors observed. Although "pollinator quality" cannot be conclusively demonstrated in terms of plant fitness after Herrera [27], judging by the combination of relative abundance and

Table 5: List of pollinator species observed more than once, in order of abundance, for the 2003 flowering season at Ham and Rice sites, at *Potentilla recta* (exotic) and *Potentilla gracilis* (native) flowers, throughout the duration of the pollinator exclusion experiment conducted at these two sites, in June and early July 2003. Bold face refers to large bodied individuals, underline refers to medium bodied individuals, and light face refers to small bodied individuals.

Pollinator species	Rice native	Pollinator species	Ham native
Hylaeus episcopalis	6	*Panurginus sp.*	23
Lasioglossum sp.	5	*Lasioglossum olympiae*	12
Trichodes ornatus	4	*Halictus ligatus*	7
Eristalis hirta	4	*Halictus tripartitus*	7
Panurginus sp.	2	*Halictus sp.*	7
Lasioglossum olympiae	2	*Apis melifera*	6
Bombus rufocinctus	2	Bombyliidae 2	6
Andrena sp.	2	*Eristalis hirta*	3
Ceratina acantha	2	***Coenonympha tullia***	3
Andrena candida	2	*Ceratina acantha*	3
Bombyliidae UID	2	*Osmia sp. A*	3
Halictus farinosus	2	*Andrena augustitarsata*	2
Chlosyne paulla	2	***Aporinellus yucatanchsis***	2
Pollenia pseudorudis	2	*Lasioglossum (Evylaeus)*	2
		Melissodes bimatris	2
17 species Seen Once	17	20 Species seen once	20
Total richness	31	Total richness	35
Total abundance	56	Total abundance	108
Pollinator species	Rice sulfur	Pollinator species	Ham sulfur
Hylaeus episcopalis	6	*Apis melifera*	27
Eristalis hirta	6	*Hylaeus episcopalis*	3
Bombus rufocinctus	2	***Bombus bifarius***	3
Bombus bifarius	2	*Eristalis hirta*	2
Andrena sp.	2	*Andrena candida*	2
Andrena prunorum	2	*Colletes sp.*	2
Ceratina acantha	2	*Osmia pusilla*	2
Megachile perhirta	2	*Andrena thaspii*	2
14 species seen once	14	16 Species seen once	16
Total richness	22	Total richness	25
Total abundance	38	Total abundance	84

the presence of cinquefoil pollen on their bodies, perhaps just seven species performed most of the pollination service during the three-year study period. Although two abundant insect species served both species of flowers (the apid *C. nanula* and the halictid *L. sisymbrii*), the European honey bee was clearly the dominant pollinator in the mix, but only for the invasive sulfur cinquefoil. Moreover, the consistent dominance of the honey bee as the principle pollinator for sulfur cinquefoil was a primary factor explaining the much higher constancy of flower visitation by potential pollinators for sulfur cinquefoil than for its native congener.

Compared to most other studies, our collection of potential pollinators was very diverse. For example, we collected 60 species of bees over the three-year study period, compared to an average of just 19.6 (± 2.5 S.E.) species of bees in pollinator surveys of single species of plants [28]. Two of these studies are worth noting here. Richards [29] found a total of only 24 species (mostly *Megachile* and *Bombus* spp.) visiting cicer milkvetch (*Astragalus cicer* L.: Fabaceae) in southern Alberta, Canada, in a similar landscape setting, with a similar sampling effort, and over a similar time period (1978 to 1981). Richards and Edwards [30] found that just six species of bees (alfalfa leafcutting bee, honey bee, and four species of *Bombus*) served as pollinators of the forage legume sainfoin (*Onobrychis viciaefolia* Scop.) in southern Alberta from June to August 1986. Interestingly, sainfoin flower-handling time was inversely correlated with pollinator body size, with bumble bees able to extract nectar at a higher rate than honey bees or leafcutting bees, and thus it is possible that glossa length, which is also correlated with body size [31], might determine whether an individual bee can successfully extract nectar from zygomorphic flowers like legumes. However, nectar within simple, open flowers like cinquefoils, can be extracted by a wide variety of insect species, including not only bees, but flies, beetles, butterflies, and wasps. The only study we could find that reported a

Table 6: Proportion of cinquefoil buds ($N = 10$) within which evidence of insect activity was observed, for 15 paired samples of *Potentilla gracilis* and *P. recta*, at Ham and Rice sites, June, 2004.

Pair no.	Site	Species	Prop. buds infested	Type of insect activity	Species	Prop. buds infested	Type of insect activity
1	Ham	*P. gracilils*	0.1	Lepidoptera exuvia	*P. recta*	0	
1	Rice	*P. gracilils*	0.4	Diptera pupae	*P. recta*	0	
2	Ham	*P. gracilils*	0.0		*P. recta*	0	
2	Rice	*P. gracilils*	0.1	Diptera pupa	*P. recta*	0	
3	Ham	*P. gracilils*	0.1	Diptera pupa	*P. recta*	0	
3	Rice	*P. gracilils*	0.0		*P. recta*	0.1	Diptera pupa
4	Ham	*P. gracilils*	0.0		*P. recta*	0	
4	Rice	*P. gracilils*	0.2	Excrement	*P. recta*	0	
5	Ham	*P. gracilils*	0.1	Unknown insect parts	*P. recta*	0	
5	Rice	*P. gracilils*	0.2	Diptera pupae	*P. recta*	0	
6	Ham	*P. gracilils*	0.0		*P. recta*	0	
6	Rice	*P. gracilils*	0.1	Unknown insect parts	*P. recta*	0	
7	Ham	*P. gracilils*	0.0		*P. recta*	0	
7	Rice	*P. gracilils*	0.5	Unknown insect parts, Diptera pupae	*P. recta*	0	
8	Ham	*P. gracilils*	0.0		*P. recta*	0	
8	Rice	*P. gracilils*	0.4	Unknown insect parts, Diptera pupae	*P. recta*	0	
9	Ham	*P. gracilils*	0.1	Unknown insect parts	*P. recta*	0	
9	Rice	*P. gracilils*	0.7	Unknown insect parts, Diptera pupae	*P. recta*	0	
10	Ham	*P. gracilils*	0.2	Unknown insect parts	*P. recta*	0	
10	Rice	*P. gracilils*	0.6	Unknown insect parts, Diptera pupae	*P. recta*	0	
11	Ham	*P. gracilils*	0.2	Unknown insect parts	*P. recta*	0	
11	Rice	*P. gracilils*	0.4	Unknown insect parts, Diptera pupae	*P. recta*	0	
12	Ham	*P. gracilils*	0.0		*P. recta*	0	
12	Rice	*P. gracilils*	0.5	Unknown insect parts	*P. recta*	0	
13	Ham	*P. gracilils*	0.0		*P. recta*	0	
13	Rice	*P. gracilils*	0.6	Unknown insect parts, Diptera pupae	*P. recta*	0	
14	Ham	*P. gracilils*	0.0		*P. recta*	0.1	Diptera pupa
14	Rice	*P. gracilils*	0.7	Unknown insect parts, Diptera pupae	*P. recta*	0	
15	Ham	*P. gracilils*	0.1	Unknown insect parts	*P. recta*	0	
15	Rice	*P. gracilils*	0.2	Unknown insect parts, Diptera pupae	*P. recta*	0.1	Diptera pupa
Mean proportion		*P. gracilils*	0.22		*P. recta*	0.01	

more diverse pollinator fauna was our own study on the flower visitors of the invasive plant yellow starthistle (*Centaurea solstitialis* L.: Asteraceae), also conducted in northeast Oregon [32], over a similar time period (2000–2002). In that study, flowers of starthistle attracted 1923 individuals and an astonishing 203 species of insects, including 87 species of bees. Compared to the present study, this is 84% more individuals, 83% more total species, and 45% more bee species, observed with a similar sampling effort. The flowers of yellow starthistle are also relatively easy to access, and are also well known to produce copious quantities of rich nectar [33], so it is likely that the combination of rich nectar and easy access explains to a large extent the richness and abundance of the pollinator fauna of yellow starthistle.

It is interesting that between 2002 and 2004, the dominant pollinator of sulfur cinquefoil in northeastern Oregon was likely to be the European honey bee. This observation lends support to the idea that sulfur cinquefoil, like yellow starthistle [11], is part of an "invasive mutualism", in which the pollinator and the plant benefit from their relationship

TABLE 7: List of species observed visiting flowers of native (*Potentialla gracilis*) and sulfur (*Potentilla recta*) cinquefoil species at four sites in northeastern Oregon, 2002, 2003, and 2004. Site Codes: MO: Morgan Lake; FH: Foothill; RI: Rice; HH: Hamburger Hill.

Order/Family/Species	Native																	Sulfur															
	MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT NAT	MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT SULF
Coleoptera	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Buprestidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Cerambycidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Cerambycidae 1	0	2	0	0	0	0	0	0	0	0	0	1	2	0	0	1	**3**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Cerambycidae 2	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	0	0	0	0	0	0	1	1	1	0	2	0	0	0	3	2	**5**
Chrysomelidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Chrysochus cobaltinus	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Chrysomelidae 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	2	0	0	0	1	0	0	1	0	0	0	2	1	1	0	**4**
Cleridae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Trichodes ornatus	1	1	0	0	0	0	13	4	6	2	1	4	2	0	23	7	**32**	0	0	1	0	0	0	5	0	0	1	0	0	1	0	5	1	**7**
Meloidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Epicauta oregana	0	0	0	0	0	0	0	1	3	0	0	0	0	0	4	0	**4**	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	0	**2**
Epicauta puncticolis	0	1	0	0	0	0	0	1	2	0	1	0	1	0	3	1	**5**	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	**2**
Lytta morenes	0	0	0	0	0	0	2	1	0	0	0	0	0	0	3	0	**3**	0	0	0	0	0	0	2	0	0	0	0	0	0	0	2	0	**2**
Coleoptera 1	0	1	0	0	0	0	0	2	5	0	0	0	1	0	7	0	**8**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Diptera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Asilidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Geron argatus	0	3	0	0	0	0	0	0	0	0	0	0	3	0	0	0	**3**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Bombyliidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Anastoechus barbatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	1	0	0	0	0	0	2	0	0	0	1	0	2	0	**3**
10 Unidentified Spp.	1	0	0	0	0	0	2	2	0	3	7	0	1	0	4	1	**15**	0	1	1	4	2	0	1	1	1	6	1	2	2	6	12	9	**29**
Calliphoridae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Pollenia pseudorudis	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2	0	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Nemestrinidae	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Sarcophagidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Blaesoxipha sp.	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	0	**2**	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	0	**2**
Sarcophagidae 1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Syrphidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Chrysotoxum fasciatum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**1**
Eristalis hirta	0	17	4	0	8	1	0	4	4	0	3	0	21	9	8	3	**41**	0	0	12	0	5	5	0	6	0	0	2	0	12	1	6	2	**30**
Parasyrphus relictus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	**2**
Platycheirus obscurus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	**1**
Spaerophoria sulphuripes	0	1	0	0	1	0	0	1	0	0	0	0	1	1	1	0	**3**	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	2	**2**
Syrphus opinator	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	0	**2**
6 Unidentified Spp.	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	**2**	0	2	0	17	4	0	2	0	0	0	1	0	2	21	2	1	**26**
Tabanidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
4 Unidentified Spp.	0	1	0	0	0	0	0	1	0	0	0	0	1	0	1	0	**2**	0	0	0	3	0	0	1	0	0	0	0	0	0	3	1	0	**4**

Table 7: Continued.

Order/Family/Species	Native																	Sulfur															
	MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT NAT	MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT SULF
Tachinidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Peleteria malleola	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	**1**
Tachinidae 1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Diptera 1	0	4	2	0	3	0	0	0	5	0	1	4	6	3	5	5	**19**	0	0	0	0	0	3	0	0	0	0	0	1	0	3	0	1	**4**
Heteroptera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Miridae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Irbisia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**1**
Hymenoptera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Andrenidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Andrena augustitarsata	0	2	0	0	0	0	0	0	0	0	2	0	2	0	0	2	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Andrena candida	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2	0	**2**	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2	**2**
Andrena miranda	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**
Andrena nigrocaerulea	0	3	0	0	0	0	0	1	0	0	0	0	3	0	1	0	**4**	0	0	0	0	0	0	0	1	1	0	0	0	0	0	2	0	**2**
Andrena pensilis	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Andrena prunorum	0	1	0	0	1	0	0	1	0	0	0	0	1	1	1	0	**3**	0	1	1	2	0	0	3	2	0	0	1	1	2	2	5	2	**11**
Andrena quintiliformis	0	1	0	0	0	0	0	1	0	0	0	0	1	0	1	0	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Andrena scurra	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	**1**	0	0	0	0	0	0	2	0	0	0	0	0	0	0	2	0	**2**
Andrena thaspii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	1	0	0	0	0	0	0	0	0	2	0	1	0	0	2	**0**
Andrena sp. 1	0	8	0	0	1	0	0	2	3	0	0	0	8	1	5	0	**14**	0	1	3	0	0	0	0	2	2	0	0	0	4	0	4	0	**8**
Andrena sp. 2	0	1	0	0	0	0	0	1	0	0	1	0	1	0	1	1	**3**	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**1**
Andrena sp. 3	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Andrena sp. 4	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Panurginus sp.	0	2	9	0	0	0	0	2	13	0	23	46	11	0	15	69	**95**	0	2	2	0	0	5	0	0	0	0	0	1	4	5	0	1	**10**
Apidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Anthophora urbana	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	**2**	0	1	0	0	0	0	0	1	0	0	0	0	1	0	1	0	**2**
Apis melifera	0	0	1	0	5	0	0	0	0	0	6	2	1	5	0	8	**14**	0	0	0	29	0	13	2	1	0	1	27	3	0	42	3	4	**76**
Bombus bifarius	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	5	0	4	0	1	3	2	0	1	3	0	5	5	5	4	**19**
Bombus californicus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	1	0	0	0	0	1	0	0	0	0	1	0	1	0	**0**
Bombus huntii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	**1**
Bombus insularis	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**1**	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	**2**
Bombus nevadensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	3	0	0	1	0	0	0	0	0	0	3	1	0	**4**
Bombus rufocinctus	0	3	0	0	1	0	0	2	0	0	1	0	3	1	2	1	**7**	0	2	2	1	0	0	3	2	3	0	1	0	4	1	8	1	**14**
Ceratina acantha	0	2	0	0	0	0	0	2	0	0	3	0	2	0	2	3	**7**	0	1	0	0	0	0	0	2	0	0	1	0	1	0	2	1	**4**
Ceratina nanula	0	0	0	0	0	0	1	1	0	39	0	0	0	0	2	39	**41**	4	1	0	8	0	0	3	0	0	9	0	0	5	8	3	9	**25**
Ceratina sequoiae	3	0	0	0	0	0	1	0	0	0	0	0	3	0	1	0	**4**	2	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	**2**
Ceratina sp. 1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	**1**	0	7	2	0	0	0	0	0	0	0	0	8	9	0	0	8	**17**
Melissodes bimatris	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Melissodes lutulenta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	**5**
Melissodes sp. 1	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	**2**	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	**1**

Table 7: Continued.

Order/Family/Species	Native																	Sulfur															
	MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT NAT	MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT SULF
Chrysididae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Chrysididae 1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Colletidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Colletes gypscolenes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	**1**
Colletes simulans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	2	0	0	0	0	0	0	0	0	0	0	0	2	0	0	**2**
Colletes sp. 1	0	1	0	0	0	0	1	0	0	0	0	0	1	0	1	0	**2**	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2	**2**
Hylaeinae	2	2	0	0	0	0	0	0	0	0	0	0	4	0	0	0	**4**	1	0	0	4	0	0	5	0	0	0	0	0	1	4	5	0	**10**
Hylaeus episcopalis	0	5	2	0	0	0	0	6	1	0	0	1	7	0	7	1	**15**	0	0	9	0	0	0	0	6	1	0	3	2	9	0	7	5	**21**
Hylaeus nunemacheri	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Crabronidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Crabronidae 1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Eumenidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Eumenidae 1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**1**	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2	0	**2**
Halictidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Agapostemon virescens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	1	**2**
Halictus farinosus	1	1	0	0	2	0	0	2	0	0	0	0	2	2	2	0	**6**	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	**2**
Halictus ligatus	0	1	4	0	0	0	0	0	1	7	7	0	5	0	1	14	**20**	0	0	1	0	0	7	0	0	3	1	1	1	1	7	3	3	**14**
Halictus sp.	0	0	0	0	0	0	0	0	2	1	7	0	0	0	2	8	**10**	0	0	0	0	0	6	1	0	0	0	0	2	0	6	1	2	**9**
Halictus tripartitus	1	3	0	0	0	0	0	1	0	0	7	0	4	0	1	7	**0**	2	3	4	5	0	0	5	1	1	3	1	0	9	5	7	4	**0**
Lasioglossum olympiae	0	7	0	0	0	0	0	2	0	0	12	0	7	0	2	12	**0**	0	2	0	0	0	0	0	1	0	0	0	0	2	0	1	0	**4**
Lasioglossum sisymbrii	3	0	0	0	0	0	2	0	0	12	0	0	3	0	2	12	**0**	0	0	0	2	2	0	8	0	0	7	0	0	0	4	8	7	**0**
Lasioglossum titusi	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**32**	1	0	0	0	1	0	2	1	0	1	1	0	1	1	3	2	**7**
Lasioglossum (Dialictus)	0	1	0	0	0	0	0	1	0	0	1	0	1	0	1	1	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Lasioglossum (Evylaeus)	0	9	0	0	0	0	0	0	0	0	2	0	9	0	0	2	**4**	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**2**
Lasioglossum sp. 1	0	1	4	0	0	0	3	5	7	0	0	6	5	0	15	6	**8**	1	0	3	4	1	1	9	0	11	5	0	3	4	6	2	8	**0**
Sphecodes sp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Ichnuemonidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Ichnuemonid 1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Megachillidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Hoplitis sp.	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	**2**	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**1**
Hoplitis albifrons	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Megachile brevis	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Megachile melanophaea	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Megachile perihirta	0	1	1	0	0	0	0	0	0	0	0	0	2	0	0	0	**2**	0	0	2	1	0	0	0	2	0	1	0	1	2	1	2	2	**7**
Megachile sp. 1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**
Osmia atrocyanea	0	2	0	0	0	0	0	0	0	0	0	0	2	0	0	0	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Osmia bridwelii	1	0	0	0	0	0	0	0	0	1	1	0	1	0	0	2	**0**	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	**0**
Osmia coloradensis	0	0	0	0	0	0	2	0	0	0	0	0	0	0	2	0	**2**	0	0	0	1	0	0	3	0	0	0	0	0	0	1	3	0	**4**
Osmia marginipennis	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Osmia pusilla	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2	**2**
Osmia (Chenosmia)	0	2	0	0	0	0	0	0	1	0	0	1	2	0	1	1	**4**	0	0	2	0	0	0	0	0	0	0	0	0	2	0	0	0	**2**

Table 7: Continued.

Order/Family/Species	Native																	Sulfur															
	MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT NAT	MO '02	MO '03	MO '04	FH '02	FH '03	FH '04	RI '02	RI '03	RI '04	HH '02	HH '03	HH '04	MO TOT	FH TOT	RI TOT	HH TOT	TOT SULF
Osmia sp. 1	0	0	2	0	0	0	0	1	1	0	1	1	2	0	2	2	**6**	0	0	2	0	0	0	0	1	0	0	0	1	2	0	1	1	**4**
Osmia sp. 2	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	3	**3**	0	2	0	0	0	0	0	0	0	0	0	0	2	0	0	0	**2**
Pompilidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Aporinellus yucatanchsis	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Sphecidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Sphex pensylvanicus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	**7**
Sphecidae sp. 1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Vespidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Polistes dominulus	0	1	0	0	1	0	0	0	0	0	0	0	1	1	0	0	**2**	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**
Stenodynerus canus	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Vespula germanica	0	1	0	0	0	0	1	0	0	0	0	0	1	0	1	0	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Vespula pennsylvanica	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	**1**
Hymenoptera 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	1	0	0	0	0	0	1	0	0	1	1	0	1	1	**3**
Hymenoptera 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**
Lepidoptera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Hesperiidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Icaricia acmon	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Lycaenidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Plebejus saepiolus	2	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	**2**	2	1	0	0	0	0	0	0	0	0	0	0	3	0	0	0	**3**
Lycaenidae 1	3	0	0	0	0	0	8	0	0	2	0	1	3	0	8	3	**14**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Nymphalidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Chlosyne paulla	1	0	0	0	0	0	0	2	0	0	0	0	1	0	2	0	**3**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Speyeria sp.	0	0	0	0	0	0	3	0	0	3	0	0	0	0	3	3	**6**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Pieridae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Colias sp.	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	7	**7**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Satyridae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Coenonympha tullia	0	1	0	0	0	0	0	1	4	0	3	4	1	0	5	7	**13**	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	**2**
Lepidoptera 1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Lepidoptera 2	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**

in an exotic location. For sulfur cinquefoil however, it is clear that a host of native insect species offer pollination service, and thus contribute to its success as an invading species. In particular, even though honeybees dominated the pollinator fauna of sulfur cinquefoil, more than 80% of flower visiting individuals were native, including more than 70 native insect species, and 46 native species of bees. Overall, the importance of native pollinators to sulfur cinquefoil indicate that this invasive is well-integrated into the ecosystem of northeastern Oregon. Moreover, although populations of sulfur cinquefoil flower for only about 45% as much time as do populations of yellow starthistle [32], this invasive cinquefoil, like starthistle, is likely to play an important role in the life histories of at least some native insect flower-visiting species.

Pollinator community constancy, reflected by temporal and spatial variance to mean ratios, was much higher for sulfur cinquefoil than for its native congener. Much of the temporal and spatial variation in flower visitors of the native cinquefoil was due to highly variable counts of some of the common bee species that frequented the native, particularly species of the smaller-bodied apid genera *Ceratina* and *Panurginus* and species of the halictid genus *Lasioglossum*. It is widely known through longer term monitoring work, that bee species such as these typically experience wide fluctuations in abundance from year to year, and from site to site within years [34, 35]. Williams et al. [28] highlighted data from several studies demonstrating that the number of "singletons" (just one observation of a species in a given study), coupled with the magnitude of spatial and temporal variation in native bee count data at the species level is typically so high that sampling efforts must be very robust to capture meaningful shifts in actual population numbers over time. However, this does not explain why sulfur cinquefoil did not tend to be serviced by so many highly variable native species during the study period, but rather tended to attract species belonging to populations that experienced much less temporal and spatial variation. In any case, this observation suggests that sulfur cinquefoil attracts a very stable pollinator fauna where it occurs in northeastern Oregon and did not seem to be limited by pollination service at any site or at any time during the study period.

Our evidence suggests that while most native insect species do not prefer sulfur cinquefoil relative to its native congener, the invasive may be a partner in an "invasive mutualism", together with the European honey bee. The honey bee was by far the most common insect observed at flowers of sulfur cinquefoil during the study period, and clearly preferred the invasive when flowers of the two cinquefoils were of equal abundance. These data are supported by the work of Barthell et al. [11], working with yellow starthistle, in which the honey bee has been implicated as an important partner in the establishment and spread of that invasive in California. Although sulfur cinquefoil can clearly reproduce by selfing (unlike yellow starthistle), the distinct response of plants to pollinator exclusion suggests that there may be a fitness consequence of selfing. In any case, this relationship of sulfur to the honey bee, and the fact that native bees, flies, and beetles did not clearly prefer sulfur cinquefoil, but visited it in accordance with its relative abundance, is consistent with observations in other systems [36, 37]. In terms of mechanisms that may explain our data on preference, it is possible that the higher sugar concentration of nectar in sulfur cinquefoil served as an attractant to honey bees. However, other qualities of nectar that we did not measure, including the ratio of sucrose to hexose [38, 39], and the presence of key amino acids [40] may be attractants as well, and may be more important for explaining why native pollinators in northeastern Oregon do not generally prefer sulfur over its native congener.

Pollinator exclusion produced a greater response in seed parameters in the invasive sulfur cinquefoil, compared to the native slender cinquefoil. The most pronounced effect was that mean seed size increased with increasingly aggressive exclusion of pollinators, at the expense of a lower seed number as pollinator exclusion became more pronounced. This supports the finding of Werner and Soule [12], who worked on the biology of sulfur cinquefoil in Michigan. However, while mean seed mass under exclosed conditions increased by only 30% in our study (two sites combined), mean mass increased by 60% in the Michigan study. The difference between the studies was even more pronounced with seed number: in northeastern Oregon, flowers produced 68% as many seeds as did open flowers, compared to just 13% for the study by Werner and Soule [12]. It seems that the kind of variation observed within and between sites in northeastern Oregon is also present when this species is studied at other geographically distant sites. Actually, variation of this kind may be more the rule than the exception, as other studies have reported similar variation and inferred its adaptive significance. For example, Kasagi and Kudo [41] reported substantial temporal variation in self-compatibility in *Phyllodoce aleutica* (Ericaceae), with high self-compatibility corresponding with periods of pollinator limitation. Werner and Soule [12] did not discuss whether the production of larger seeds had any adaptive significance for sulfur cinquefoil, or whether seed size increase is merely a consequence of a change in the rate of seed production, induced by the lack of pollen at a critical time in development. In any case, we observed no difference in germination rate for the larger seeds produced in the bagged treatment. Additional research on the fate of fertilized seedlings, versus those produced by selfing, would be needed to establish the conditions under which selfing might be advantageous.

Compared to pollinator exclusion studies on more self-incompatible plant species (e.g., yellow starthistle; [11]), the magnitude of our results were subtle. Yellow starthistle responded to pollinator exclusion by producing very few seeds in the exclosed condition, lending support to the idea that pollinators such as honey bees are indeed "invasive mutualists" and tend to facilitate invasion of some exotic species. While it is clear that sulfur cinquefoil can produce viable seeds without fertilization, it is interesting nonetheless that this invasive species is markedly more responsive to pollinator exclusion than is its native congener.

In terms of seed biology, we observed three differences between sulfur cinquefoil and its native congener: for sulfur, the general lack of evidence of seed predation, the greater

allocation to seed production relative to vegetative biomass, and a much more prolonged germination sequence, lasting nearly a year. First, one of the best documented observations on invasive species is the lack of effective natural enemies in the first decades of invasion [42]. Our observations on seed predation support the idea that native seed predators have not had sufficient time to adapt to the smaller sulfur cinquefoil seeds since introduction occurred a little more than 100 years ago. Indeed, although we did not establish a causal connection between the magnitude of seed predation and germination rates, it is noteworthy that proportionally nearly twice as many sulfur seeds germinated as did the native. Second, sulfur cinquefoil dedicated three times as much energy to reproduction each year during the three-year study period as did the native cinquefoil. It has long been observed that ruderal plant species tend to allocate proportionally greater resources to reproduction, even under relatively stressful environmental conditions [43, 44]. This strategy seems to balance the increased risk of mortality in the parent, with the increased opportunity for survival of the offspring. Similarly, the much longer germination "window" observed in sulfur cinquefoil, relative to the native, may be a strategy for retaining opportunity to take advantage of disturbed habitats over a longer period of time. Sulfur cinquefoil is highly successful at "filling in" suitable habitat once it arrives on the scene [45]. A longer germination window may be one mechanism this invasive species uses to gradually occupy an area once it colonizes. The native cinquefoil species on the other hand, can only respond to disturbance in a previously colonized area within a short period of time each year (~2 months), and thus may be at a competitive disadvantage over the long run, where it cooccurs with sulfur cinquefoil.

In general, our comparative data indicate that the invasive sulfur cinquefoil and the native slender cinquefoil employ different adaptive strategies, with the invasive using more of a "ruderal" strategy, as opposed to a "stress-tolerant" strategy used by the native [44]. Sulfur cinquefoil is clearly preferred as a nectar source by honey bees, utilizes a suite of native pollinators as well, invests relatively more energy in seed production, and enhances its chances to seize opportunities for disturbed conditions over a much longer period of time relative to the native cinquefoil. While our observations underline key differences in life history between sulfur cinquefoil and its native congener, additional work is required to understand exactly how these differences may translate into fitness differentials in the long run.

Acknowledgments

This project was funded by the U.S. Forest Service Pacific Northwest Research Station and benefited from the guidance of two Station scientists, Dr. Jane Hayes and Dr. Catherine Parks. Bees were identified by Dr. Robbin Thorp (University of California, Davis USA); all other insects were identified by the Systematic Entomology Laboratory (USDA) in Beltsville, MD. Assistance with the identification of plants was provided by Marti Aitken and Bridgett Read Naylor. Field and laboratory assistance was provided by Euell Macke, James Stolon, Christine Powell, Evan Squire, and Nick Clavato.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 646740, 13 pages
doi:10.1155/2012/646740

Review Article

Botanicals as Grain Protectants

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Received 12 April 2012; Revised 8 June 2012; Accepted 14 June 2012

Academic Editor: John T. Trumble

Prevention of food losses during postharvest storage is of paramount economic importance. Integrated pest management is now a widely accepted strategy in pest control including postharvest infestation control which involves the use of chemical (contact/residual) insecticides along with fumigants. The use of synthetic chemical insecticides is either not permitted or used restrictively because of the residue problem and health risks to consumers. In view of the above, there is a need for plants that may provide potential alternatives to the currently used insect control agents as they constitute a rich source of bioactive molecules. Available literature indicates that plant could be source for new insecticides. Therefore, there is a great potential for a plant-derived insecticidal compounds. This paper focuses on the current state of the botanical insecticides as grain protectants and its mode of action.

1. Introduction

Food grain losses due to insect infestation during storage are a serious problem, particularly in the developing countries [1, 2]. Losses caused by insects include not only the direct consumption of kernels, but also accumulation of exuviae, webbing, and cadavers. High levels of the insect detritus may result in grain that is unfit for human consumption and loss of the food commodities, both, in terms of quality and quantity. Insect infestation-induced changes in the storage environment may cause warm moist "hotspots" that provide suitable conditions for storage fungi that cause further losses. It is estimated that more than 20,000 species of field and storage pests destroy approximately one-third of the world's food production, valued annually at more than $100 billion among which the highest losses (43%) occurring in the developing world [3, 4]. The quantitative and qualitative damage to stored grains and grain product from the insect pests may amount to 20–30% in the tropical zone and 5–10% in the temperate zone [5, 6]. Food grain production in India has reached 250 million tonnes in the year 2010-2011, in which nearly 20–25% food grains are damaged by stored grain insect pests [7, 8]. The efficient control and removal of stored grain pests from food commodities has long been the goal of entomologists throughout the world.

The major pests of stored grain and pulses of the Indian subcontinent are classified in to two groups, namely, primary pests: those which are capable of penetrating and infesting intact kernel of grain and have immature stages develop within kernel of grain and secondary pests which cannot infest the whole grain but feed on as broken kernels, debris, high moisture weed seeds, and grain damaged by primary pests. In general, the immature stages of the secondary pest species are found external to the grain. It is often thought that secondary invaders cannot initiate infestation. The important primary pests are the rice weevil, *Sitophilus oryzae* (L.), granary weevil, *Sitophilus granaries* (L.), (Coleoptera: Curculionidae), lesser grain borer, *Rhyzopertha dominica* (F.), (Coleoptera: Bostrichidae), Khapra beetle, *Trogoderma granarium* (Everts), (Coleoptera: Dermestidae), and the pulse beetle *Callosobruchus chinensis* (L.) (Coleoptera: Bruchidae). The secondary pests are rust-red flour beetle, *Tribolium castaneum* (Herbst), (Coleoptera: Tenebrionidae), rusty grain beetle, *Cryptolestes ferrugineus* (L.), (Coleoptera: Cucujidae),

sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.), (Coleoptera: Silvanidae), mites, (Acarina: Tetranychidae) *Liposcelis corrodens*, and (Psocoptera: Liposcelidae).

2. Infestation Control by Pesticides and Their Side Effects

Since the 1950s, synthetic insecticides have been used extensively in grain facilities to control stored product insect pests. Fumigants such as methyl bromide, phosphine, cyanogens, ethyl formate, or sulfuryl fluoride rapidly kill all life stages of stored product insects in a commodity or in a storage structure. Fumigation is still one of the most effective methods for the prevention of stored product losses from insect pests. But pests develop resistance, not stored products were showing a slow upsurge in fumigation resistance [9]. Resistance to phosphine is so high in Australia and India, it may cause control failures [10, 11]. Methyl bromide has been identified as a major contributor to ozone depletion [12] and has been banned in developed countries, and developing nations have committed to reducing the use by 20% in 2005 and phase out in 2015. Contact insecticides such as malathion, chlorpyrifos, or deltamethrin are sprayed directly on grain or storage structure for protection from infestation for several months. The incidence of insecticide resistance is a growing problem in stored-product protection. Resistance to one or more insecticides has been reported in at least 500 species of insects and mites [13].

Champ [14] reported that resistance to pesticides used to protect grain and other stored food stuffs is widespread and involves all groups of pesticides and most of the important pests. Some of the contact insecticides have become ineffective because of widespread resistance in insect population. Resistance to malathion is widespread in Canada, USA and Australia [15]. Stored product insects pests were found to be resistant against different insecticides including the cyclodienes, chlorpyrifos, cyanophos, carbamates, carbaryl, cypermethrin, dichlorodiphenyltrichloroethane, deltamethrin, diazinon, dichlorovos, ethylene bromide, ethyl formate organophosphates, permethrin, pyrethrins, and propoxur.

Although chemical insecticides are effective, their repeated use has led to residual toxicity, environmental pollution and an adverse effect on food besides side effect on humans [16, 17]. Their uninterrupted and indiscriminate use not only has led to the development of resistant strains but also accumulation of toxic residues on food grains used for human consumption that has led to the health hazards [18]. In view of all these problems, several insecticides have either been banned or restricted in their use.

3. Botanicals as Alternative to Synthetic Pesticides

The increasing serious problems of resistance and residue to pesticides and contamination of the biosphere associated with large-scale use of broad spectrum synthetic pesticides have led to the need for effective biodegradable pesticides with greater selectivity. This awareness has created a worldwide interest in the development of alternative strategies, including the discovery of newer insecticides [19, 20]. However, newer insecticides will have to meet entirely different standards. They must be pest specific, nonphytotoxic, nontoxic to mammals, ecofriendly, less prone to pesticide resistance, relatively less expensive, and locally available [21]. This has led to re-examination of the century-old practices of protecting stored products using plant-derivatives, which have been known to resist insect attack [5, 22–24]. Plant derived materials are more readily biodegradable, less likely to contaminate the environment and nay be less toxic to mammals. There are many examples of very toxic plant compounds. Therefore, today, researchers are seeking new classes of naturally occurring insecticides that might be compatible with newer pest control approaches [2, 25, 26].

Since ancient times, there have been efforts to protect harvest production against pests. The Egyptian and Indian farmers used to mix the stored with fire ashes [83, 84]. The ancient Romans used false hellebore (*Veratrum album*) as a rodenticide, the Chinese is credited with discovering the insecticidal properties of Derris species, whereas pyrethrum was used as an insecticide in Persia and China [4]. In many parts of the world, locally available plants are currently in wide use to protect stored products against damage caused by insect infestation [80, 85–87]. Indian farmers used neem leaves and seed for the control of stored grain pests [88]. In northern Cameroon, cowpeas are traditionally mixed with sieved ash after threshing and the mixture put into mud granaries or clay jars [89]. In eastern Africa, leaves of the wild shrub *Ocimum suave* and the cloves of *Eugenia aromatic* are traditionally used as stored grain protectants [90]. In Rwanda, farmers store edible beans in a traditional closed structure (imboho) and whole leaves of *Ocimum canum* are usually added to the stored foodstuff to prevent insect damage within these structures [75]. Owusu [91] suggested natural and cheaper methods for the control of stored product pests of cereals, with traditionally useful Ghanaian plant materials. In some south Asian countries, food grains such as rice or wheat are traditionally stored by mixing with 2% turmeric powder [92, 93]. The use of oils in stored-products pest control is also an ancient practice. Botanical insecticides such as pyrethrum, derris, nicotine, oil of citronella, and other plant extracts have been used for centuries [27, 94, 95]. More than 150 species of forest and roadside trees in India produce oilseeds, which have been mainly used for lighting, medicinal purposes, and also as insecticides from ancient times to early 20th century [96]. Turmeric, garlic, *Vitex negundo*, gliricidia, castor, *Aristolochia*, ginger, *Agave americana*, custard apple, *Datura*, *Calotropis*, *Ipomoea,* and coriander are some of the other widely used botanicals to control and repel crop pests [81, 97].

Talukder [5] has listed 43 plant species as insect repellents, 21 plants as insect feeding deterrents, 47 plants as insect toxicants, 37 plants as grain protectants, 27 plants as insect reproduction inhibitors, and 7 plants as insect growth and development inhibitors. Eighteen species showed insecticidal

potential, and antiovipositional properties against *Sitophilus oryzae* [98].

4. Classification of Botanical Insecticides

On the basis of physiological activities on insects, Jacobson [3] conventionally classified the plant components into 6 groups, namely, repellents, feeding deterrents/antifeedants, toxicants, growth retardants, chemosterilants, and attractants. Focus on the toxicants and grain protectants on activity of essential oil, extracts, and its constituents has sharpened since the 1980s.

4.1. Repellents. The repellents are desirable chemicals as they offer protection with minimal impact on the ecosystem, as they drive away the insect pest from the treated materials by stimulating olfactory or other receptors. Repellents from plant origins are considered safe in pest control; minimise pesticide residue; ensure safety of the people, food, and environment [1, 5, 99]. The plant extracts, powders, and essential oil from the different bioactive plants were reported as repellent against stored grain insect pests [1, 91, 100–102]. For example, the essential oil of *Artemisia annua* was found as repellent against *Tribolium castaneum* and *Callosobruchus maculates* [103].

4.2. Antifeedants/Feeding Deterrents. Antifeedants, sometimes referred to as "feeding deterrents" are defined as chemicals that inhibit feeding or disrupt insect feeding by rendering the treated materials unattractive or unpalatable [104, 105]. Some naturally occurring antifeedants, which have been characterized, include glycosides of steroidal alkaloids, aromatic steroids, hydroxylated steroid meliantriol, triterpene hemiacetal, and others [3, 106]. Essential oil constituents such as thymol, citronellal and α-terpineol are effective as feeding deterrent against tobacco cutworm, *Spodoptera litura* synergism, or additive effects of combination of monoterpenoids from essential oils have been reported against *Spodoptera litura* larvae [107]. The screening of several medicinal herbs showed that root bark of *Dictamnus dasycarpus* possessed significant feeding deterrence against two stored-product insects [108].

4.3. Toxicants. Research on new toxicants of plant origin has not declined in recent years despite the increased research devoted to the discovery of synthetic insecticides [25]. Worldwide reports on plant derivates showed that many plant products are toxic to stored product insects [6, 16, 27, 55, 82, 91, 109–114]. Talukder [32] listed the use of 43 plant species expressing toxicant effects of different species of stored-products insects. Pascual-Villalobos and Robledo [115] carried out screening of plant extracts from 50 different wild plant species of southeastern Spain for insecticidal activity towards *Tribolium castaneum* and reported that four species, namely, *Anabasis hispanica, Senecio lopezii, Bellardia trixago,* and *Asphodelus fistulosus* were found be promising. Two major constituents of the essential oil of garlic, *Allium sativum*, methyl allyl disulfide and diallyl trisulfide were to be potent toxicant and fumigants against *Sitophilus zeamais* and *Tribolium castaneum* [116]. Rahman [117] reported that nicotine, an active component of *Nicotiana tabacum,* is a strong organic poison which acts as a contact-stomach poison with insecticidal properties. This compound is, of course, very toxic to humans as well. The essential oil vapours distilled from anise, cumin, eucalyptus, oregano, and rosemary were also reported as fumigantants and caused 100% mortality of the eggs of *Tribolium confusum* and *Ephestia kuehniella* [118]. Many species of the genus *Ocimum* oils, extracts, and their bioactive compounds have been reported to have insecticidal activities against various insect species [59, 119]. A list of many known toxicants from plant origin, reported as effective on stored-product insect-pest management, is given in Table 1.

4.4. Natural Grain Protectants. From very early times, plant materials have been used as natural protectants of stored grains. Worldwide reports indicate that when mixed with stored grains, leaf, bark, seed powder, or oil extracts of plants reduce oviposition rate and suppress adult emergence of stored product insects, and also reduce seed damage rates [25, 40, 46, 87, 119–122]. In 1989, Jacobson [123] noted that the most promising natural grain protectants were generally observed in the plant families, Annonaceae, Asteraceae, Canellaceae, Labiatae, Meliaceae, and Rutaceae.

The Indian neem plant is the most well-known example and its various parts, namely, leaves, crushed seeds, powdered fruits, oil, and so forth, have been used to protect stored grain from infestation [1, 124, 125]. The neem oil and kernel powder gave effective grain protection against stored grain insect pests like *Sitophilus oryzae, Tribolium cataneum, Rhyzopertha dominica,* and *Callosobruchus chinensis* at the rate of 1 to 2% kernel powder or oil [126]. The neem oil adhered to grain forms uniform coating around the grains against storage pests for a period of 180–330 days [127]. Yadava and Bhatnagar [128] reported that a dried leaves of *Azadirachta indica* have been mixed with stored grains for protection against insects. Azadirachtin is an active principle from the neem plant, which is an effective grain protectant against insect infestation [129]. Rajashekar et al. [7] reported that root powder extracts of *Decalepis hamiltonii* have been mixed with stored grains for protection against various stored grain insect pests. Eighteen species offered protection to wheat up to 9 months without affecting seed germination [98].

In parts of eastern Africa, leaves of some plants and allelochemicals including azadirachtin, nicotine, and rotenone have traditionally been used as grain protectants [5, 130]. The powders of *Rauvolfia serpentina, Acorus calamus,* and *Mesua ferrea* are used as a grain protectant against *Rhyzopertha dominica* [131]. In a survey in northern semiarid regions of Ghana only 16 plants were identified as being used as grain protectants [132]. In Africa, the grain protectant potential of different plant derivatives, including plant oils against major stored-product pests were also found to be very promising and reduced the risks associated with the use of insecticides [82, 121]. In northern Cameroon, the

Table 1: List of plant species reported to show insecticidal activity.

Plant species	Family	Plant part	References
Acorus calamus	Acoraceae	O, RO	[27]
Allium sativum	Alliaceae	P	[28]
Annona squamosa	Annonaceae	L	[29]
Aphanamixis polystachya	Meliaceae	SC, SE	[25]
Azadirachta indica	Meliaceae	O, SP, LP	[30]
Baccharis salicifolia	Asteraceae	O	[31]
Bassia longifolia	Sapotaceae	E	[5]
Brassica spp.	Cruciferae	L, ZE	[32]
Cajanus cajan	Fabaceae	O	[33]
Calophyllum inophyllum	Clusiaceae	O	[34]
Calotropis procera	Apocynaceae	LP	[35]
Carum carvi	Apiaceae	FE	[36]
Cinnamomum aromaticum	Lauraceae	B	[37]
Citrus	Rutaceae	O	[38]
Curcuma longa	Zingiberaceae	P	[39]
Chenopodium ambrosioides	Amaranthaceae	FE, O	[40]
Cocos nucifera	Arecaceae	O	[25]
Convolvulus arvensis	Convolvulaceae	LE	[41]
Conyza dioscoridis	Asteraceae	ZE	[41]
Coriandrum sativum	Apiaceae	SE, O	[42]
Datura alba	Solanaceae	LP	[43]
Decalepis hamiltonii	Asclepiadaceae	XP	[7]
Eichhornia crassipes	Pontederiaceae	LE	[44]
Elaeis guineensis	Arecaceae	O	[45]
Elaeis guineensis	Palmaceae	O	[46]
Embelia ribes	Myrsinaceae	FE, O	[47]
Eucalyptus globules	Myrtaceae	LP, M	[48]
Foeniculum vulgare	Apiaceae	FE	[49]
Glycine max	Fabaceae	O	[50]
Jatropha gossypifolia	Euphorbiaceae	SE	[51]
Juniperus virginiana	Cupressaceae	O	[52]
Lantana camara	Verbenaceae	TE	[45]
Lonchocarpus spp.	Leguminosae	O	[53]
Lupinus albus	Fabaceae	SE	[54]
Lupinus termis	Leguminosae	SE	[54]
Melia azedarach	Meliaceae	O, E	[55]
Mentha citrate	Lamiaceae	O	[56]
Nicotiana tabacum	Solanaceae	E	[57]
Ocimum canum	Lamiaceae	LP	[58]
Ocimum kilimandscharicum	Lamiaceae	O	[59]
Piper nigrum	Piperaceae	O, E	[60, 61]
Polygonum hydropiper	Polygonaceae	L	[62]
Pongamia glabra	Fabaceae	O, E	[61]
Psidium guajava	Myrtaceae	L, LP	[63]
Ryania speciosa	Flacourtiaceae	YE	[64]
Sapindus trifoliatus	Sapindaceae	SP	[65]
Schleichera trijuga	Sapindaceae	O	[66]
Sesamum orientale	Pedaliaceae	O	[5]
Sesamum indicum	Pedaliaceae	O	[67]
Syzygium aromaticum	Myrtaceae	O	[68]
Tagetes erecta	—	X, Y	[69]
Tanacetum cinerariaefolium	Asteraceae	O, P	[55]
Thujopsis dolabrata	Cupressaceae	E	[64]
Trigonella foenumgraecum	Fabaceae	SE	[70]
Vitex negundo	Lamiaceae	L	[71]

Note. L: leaves, B: bark, F: fruits, S: seeds, O: oil, P: powder, E: extract, M: vapour, R: Rhizome, T: plant, V: flower, X: root, and Y: stem, (Source: [5, 6]).

essential oils of plants *Xylopia aethiopica, Vepris heterophylla,* and *Luppia rugosa* are used for protection of stored grains from attack of stored grain insect pests [114]. The components of citrus peels were used as grain protectant against *Callosobruchus maculatus* [133]. Coconut oil has been found effective against *Callosobruchus chinensis,* for a storage period of six months, when applied to *Vigna radiata* (green gram) at 1% [134]. Formulations of menthol were used as protection of pulse grain from attack of *Callosobruchus Chinensis* [135]. Spinosad, a naturally occurring insecticide from the actinomycete, *Saccharopolyspora spinosa,* has high efficacy, a broad insect pest spectrum, low mammalian toxicity, and minimal environmental profile is unique among existing products currently used for stored-grain protection [136].

4.5. Chemosterilants/Reproduction Inhibitors. Many researchers reported that plant parts, oil, extracts, and powder mixed with grain-reduced insect oviposition, egg hatchability, postembryonic development, and progeny production [137–139]. Lists of 43 plant species have been reported as reproduction inhibitors against stored product insects [32]. Reports have also indicated that plant derivatives including the essential oils caused mortality of insect eggs [82]. Many ground plant parts, extracts, oils, and vapour also suppress many insects [6, 7].

4.6. Insect Growth and Development Inhibitors. Plant extracts showed deleterious effect on the growth and development of insects and reduced larval pupal and adult weight significantly, lengthened the larval and pupal periods, and reduced pupal recovery and adult eclosion [140]. Rajasekaran and Kumaraswami [141] reported that grains coated with plant extracts completely inhibited the development of insect

Table 2: List of insecticidal active principles of plants.

Active principle	Plant species	Insect toxicity	Insect species	References
Anonaine	*Annona reticulate*	Contact	*Callosobruchus chinensis*	[72]
Azadirachtin	*Azadirachta indica*	Contact: IGR	Stored grain pests, aphids	[30]
E-Anethole	*Foeniculum vulgare*	Contact	*Sitophilus oryzae*, *Callosobruchus chinensis*	[49]
β-Asarone	*Acorus calamus*	Contact;	Stored grain pests	[73]
Z-Asarone	*Acorus calamus*; *Acorus gramineus*	Contact	*Sitophilus zeamais*	[26]
Bornyl acetate	*Chamaecyparis obtuse*	Contact	*Sitophilus oryzae*	[27]
Camphor	*Ocimum kilimandscharicum*	Contact	*Sitophilus oryzae*	[59]
(+)-3-Carene	*Baccharis salicifolia*	Contact	*Tribolium castaneum*	[59]
Carvacrol	*Thujopsis dolabrata*	Contact; fumigant	*Sitophilus oryzae*, *Callosobruchus chinensis*	[60]
Carvone	*Carum carvi*	Contact	*Sitophilus oryzae*, *Rhyzopertha dominica*	[74]
1,8 Cineole	*Eucalyptus*	Contact; fumigant	*Rhyzopertha dominica* *Tribolium castaneum*	[38]
Cinnamaldehyde	*Cinnamomum aromaticum*	Contact	*Tribolium castaneum*, *Sitophilus zeamais*	[37]
Dioctyl hexanedioate	*Conyza dioscoridis*	Contact	*Tribolium castaneum*, *Sitophilus granaries*	[41]
Eugenol	*Citrus*	Fumigant	*Sitophilus oryzae*	[38]
Estragole	*Foeniculum vulgare*	Contact	*Sitophilus oryzae* *Lasioderma serricorne*	[31]
(+)-Fenchone	*Foeniculum vulgare*	Contact	*Sitophilus oryzae* *Lasioderma serricorne*	[31]
Hexa decane	*Chenopodiumambrosioides*	Contact	*Tribolium castaneum*, *Sitophilus granaries*	[41]
Hexadecanoic acid	*Convolvulus arvensis*	Contact	*Sitophilus oryzae*, *Rhyzopertha dominica.*	[41]
Linalool	*Ocimum canum* Sims	Fumigant	*Tribolium castaneum*, *Sitophilus granaries*	[75]
Limonene	*Citrus*	Contact	*Tribolium castaneum*	[27]
(−)-Limonene	*Baccharis salicifolia*	Contact; fumigant	*Tribolium castaneum*	[31]
Nicotine	*Nicotiana tabacum*	Contact	Mites, aphids, thrips, leafhopper	[39]
Pyrethrin I and II	*Tanacetum cinerariaefolium*	Contact; stomach poison	Stored grain pests, crop pests	[76]
β-Pinene	*Baccharis salicifolia*	Contact	*Tribolium castaneum*,	[27]
α-Pinene	*Baccharis salicifolia*	Fumigant	*Tribolium castaneum*,	[27]
Rotenone	*Lonchocarpus* sp.	Contact; stomach poison	Crop pests, lace bugs, *Sitophilus oryzae*	[55]
Ryania	*Ryania speciosa*	Contact; stomach poison	Potato beetle, aphids, lace bugs, stored grain pests	[77]
Sabadilla	*Schoenocaulon officinale*	Contact; stomach poison	Stinks, thrips, squash bugs, leaf hoppers, caterpillars	[78]
Spinosyn A and D	*Saccharopolyspora spinosa*	Stomach poison	Stored grain pests	[79]

like *Sitophilus oryzae*. Plant derivatives also reduce the survival rates of larvae and pupae and adult emergence [101]. Development of eggs and immature stages inside grain kernel were also inhibited by plant derivatives [102]. The crude extract also retarded development and caused mortality of larvae, cuticle melanisation, and high mortality in adults [142].

5. Some Important Phytochemicals with Insecticidal Properties

The botanical insecticides that have primarily been used and are commercially available include ryania, rotenone, pyrethrin, nicotine, azadirachtin, and sabadilla (Tables 2 and 3).

Table 3: Insecticidal activity and mammalian toxicity of some natural insecticides.

Natural insecticides	Insect toxicity*	Mammalian toxicity Oral (rat) LD_{50} (mg/kg b.w.)
Anethole	C, S	2090
β-asarone	C, S	275
Azadirachtin	IGR, R	13000
Carvacrol	C	810
1,8-Cineole	C, F	2480
Cinnamaldehyde	C	1160
Cuminic aldehyde	C, S	1390
Eugenol	C, F	500
Nicotine	C	50
Pyrethrin I and II	C, S	1200
Rotenone	S	350
Ryania	C, S	750
Sabadilla	C, S	5000
Spinosad	C	3738

*C: contact, S: stomach poison, F: fumigant, IGR: insect growth regulator, and R: repellent [80–82].

5.1. Ryania. The active components of ryania are derived from the roots and woody stems of the plant *Ryania speciosa*, native to Trinidad [143]. Ryania has low mammalian toxicity, with a median lethal dose (LD_{50}) of 750 mg/kg and works as both contact and stomach poison. It has long residual activity among the botanical insecticides. This botanical insecticide has a unique mode of action and affects muscles by binding to the calcium channels in the sarcoplasmic reticulum. This causes calcium ion flow into the cells, and death follows very rapidly [20]. Ryania works best on caterpillars (i.e., codling moth, corn earworm); however, is it also active on a wide range of insects and mites, including potato beetle, lace bugs, aphids and squash bug [144].

5.2. Rotenone. Rotenone is derived from the roots of the two plants: *Lonchocarpus* sp. and *Derris* sp. are both legumes originally from the East Indies, Malaya and South America. Rotenone is a moderately of the toxic botanical insecticides, with an LD_{50} of 132 mg/kg to mammals [81]. In fact, rotenone is more toxic to mammals than both carbaryl and malathion, two commonly used synthetically derived insecticides. Also, rotenone is extremely toxic to fish [55]. This botanical insecticide works as both contact and stomach poison. Rotenone is slower acting than most other botanical insecticides, taking several days to kill pests; however, pests stop feeding almost immediately. It degrades rapidly in air and sunlight. Rotenone blocks respiration by electron transport on the complex I. Rotenone shows broad spectrum of activity on many insects and mite pests, including leaf-feeding beetles, caterpillars, lice, mosquitoes, ticks, fleas, and fire ants [145].

5.3. Pyrethrin/Pyrethrum. Pyrethrin I and II are derived from the seeds or flower of *Chrysanthemum cinerariaefolium* [55, 146] which is grown in Africa, Ecuador, and Kenya. Pyrethrin has a low mammalian toxicity. However, cats are highly susceptible to pyrethrin poisoning. The LD_{50} of pyrethrin is 1200 to 1,500 mg/kg [81, 147, 148]. Pyrethrin is one of the oldest household insecticides still available and is fast acting, providing almost immediate "knockdown" of insects following an application. It works as both a contact and a stomach poison. The material has a very short residual activity-degrading rapidly under sunlight, air and moisture, which means that frequent applications may be required. Pyrethrin can be used up until harvest, as there is no waiting interval required between initial application and harvest of food crops [149].

The way pyrethrin kills insects (mode of activity) is by disrupting the sodium and potassium ion-exchange process in insect nerves and interrupting the normal transmission of nerve impulses. Pyrethrin has activity on wide range of insects and mites, including flies, fleas, beetles, and spider mites [150].

5.4. Nicotine. Nicotine, which is derived from *Nicotiana tabacum*, is toxic to mammals among the botanical insecticides with an LD_{50} between 50 and 60 mg/kg [55, 151]. It is extremely harmful to humans. Nicotine, a fast-acting nerve toxin, works as a contact poison. It kills insects (and humans) through bonding to receptors at the nerve synapses (junctures), causing uncontrolled nerve firing, and by mimicking acetylcholine (Ach) at the nerve-muscle junctions in the central nervous system [152].

Certain plant types, such as roses, may be harmed or injured by nicotine sprays. Nicotine is most effective on soft-bodied insects and mites, including aphids, thrips, leafhoppers, and spider mites. Many caterpillars are resistant to nicotine [153].

5.5. Azadirachtin. Azadirachtin is derived from the tree *Azadirachta indica*, grown in India and Africa [55]. Azadirachtin has an extremely low mammalian toxicity and is least toxic of the commercial botanical insecticides, with an LD_{50} of 13,000 mg/kg. Azadirachtin is considered a contact poison; however, it has "some" systemic activity in plants when applied to the foliage. The material is generally nontoxic to beneficial insects and mites. Azadirachtin has broad mode of activity, working as a feeding deterrent, insect-growth regulator, repellent, and sterilant; and it may also inhibit oviposition [55, 154]. The material is active on a broad range of insects, including stored grain pests, aphids, caterpillars and mealybugs [30].

5.6. Sabadilla. Sabadilla is derived from the seeds of plant *Schoenocaulon officinale*, which is grown in Venezuela. Sabadilla is one of the least toxic registered botanical insecticides, with mammalian LD_{50} of 5,000 mg/kg. Sabadilla works as contact toxicant and a stomach poison. Similar to other botanical insecticides, the material has minimal residual activity and degrades rapidly in sunlight and moisture

(rainfall). Sabadilla works by affecting nerve cell membranes, causing loss of nerve function, paralysis, and death [146]. It is effective against caterpillars, leaf hoppers, thrips, stink, and squash bugs.

5.7. Avermectins. Avermectins, which are macrocyclic lactones, are derived from the actinomycete, *Streptomyces avermitilis* [155], lethal dosage of 50% in range of 10–11.3 mg/kg for rat. This molecule is most effective against agricultural pests with lethal concentration of 90% (LC_{90}) in the range of 0.02 ppm for mites and has somewhat least toxicity to stored products pests. It is effective on internal parasites of domestic animals [156]. Avermectins block the neurotransmitter GABA at the neuromuscular junction in insects and mites. Visible activity, such as feeding and egg laying, stops shortly after exposure, though death may not occur for several days [157].

5.8. Spinosads. Spinosad is a mixture of spinosyn A and spinosyn D and was originally isolated from the soil Actinomycete, *Saccharopolyspora spinosa* [158]. Spinosad is recommended for the control of a very wide range of caterpillars, leaf miners and foliage-feeding beetles. Spinosyns have a novel mode of action, primarily targeting binding sites on nicotinic acetylcholine receptors that are distinct from those at which other insecticides exert activity, leading to disruption of acetylcholine neurotransmission [79, 159].

5.9. (Z) Asarone. (Z) Asarone is natural insecticide isolated from *Acorus calamus* L. [26]. This molecule is more effective against adults of *Sitophilus oryzae, Lasioderma serricorne,* and *Callosobruchus chinensis* and shows both fumigant and contact toxicity. Some studies show that the molecules possess *in vivo* carcinogenic effects [160] and *in vitro* mutagenic activites [161]. Further, this molecule induces structural chromosome aberration in human lymphocytes *in vitro* [162]. Due to its mammalian toxicity [81, 163], the molecule is unsafe for grain treatment.

6. Challenges to the Utilization of Botanicals Pesticides

Many plant species contain secondary metabolites that are potent against several pest species. Not only are some of the plants (e.g., the neem trees) of major interest as sources of phytochemicals for more environmentally benign grain/crop protection. Phytochemical products can increase income of rural farmers and promote safety and quality of food and life in general [8, 164].

The successful utilization of botanicals can help to control many of the world's destructive pests and diseases, as well as reduce erosion, desertification, deforestation, and perhaps even reducing human population by acting as spermaticide (although this will be considered a major negative effect by many cultures and religions) [165]. Although the possibilities of using botanical pesticides seem almost endless, many details remain to be clarified. Many obstacles must be overcome and many uncertainties clarified before their potential can be fully realized. These limitations seem surmountable; however, they present exciting challenges to the scientific and economic development communities. Solving the following obstacles and uncertainties may well bring a major new resource which will benefit much of the world. These obstacles include:

(i) lack of experience and appreciation of the efficacy of botanicals for pest control. There are still doubts as to the effectiveness of plant-derived products (both "home-made" and commercial products) due to their slow action and lack of rapid knockdown effect;

(ii) genetic variability of plant species in different localities;

(iii) difficulty of registration and patenting of natural products and lack of standardization of botanical pesticide products;

(iv) economic uncertainties occasioned by seasonal supply of seeds, perennial nature of most botanical trees, and change in potency with location and time with respect to geographical limitations;

(v) handling difficulties as there is no method for mechanizing the process of collecting, storing, or handling the seeds or leaves or flowers from some of the perennial trees;

(vi) instability of the active ingredients when exposed to direct sunlight;

(vii) competition with synthetic pesticides through aggressive advertising by commercial pesticides dealers and commercial-formulated botanicals are more expensive than synthetic insecticides and are not as widely available;

(viii) rapid degradation, although desirable in some respects, creates the need for more precise timing or more frequent applications;

(ix) Data on the effectiveness and long-term (chronic) mammalian toxicity are unavailable for some botanicals, and tolerances for some have not been established.

7. Conclusion

Many authors have evaluated the insecticidal (grain protectant) properties of plant products on various species of stored product insect pests. The results clearly show that it is possible to develop methods for grain protectants with reduced use of synthetic chemical insecticides. The main advantages of botanical pesticides are ecofriendly, easily biodegradable, nontoxic to nontarget organisms, and many plant-derived natural products acting against insects could be produced from locally available raw materials. They have been numerous botanical pesticides studied at the laboratory level. Research efforts should focus not only on their efficacy, but also on mammalian toxicity, mode of action in insects, seed germination, effect on nutritional quality, seedling growth, and stability of the compound. The insecticides of

plant origin could be exploited for the development of novel molecules with highly precise targets for sustainable insect pest management in stored grain.

Acknowledgments

The authors thank the Director, NBAII, Bangalore, for encouragement and support. The first author acknowledges the Department of Biotechnology, New Delhi, for awarding the postdoctoral research associateship.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 125284, 5 pages
doi:10.1155/2012/125284

Research Article

Investigations on the Effects of Five Different Plant Extracts on the Two-Spotted Mite *Tetranychus urticae* Koch (Arachnida: Tetranychidae)

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Received 5 April 2012; Accepted 18 June 2012

Academic Editor: Kabkaew Sukontason

Two-spotted mite, *Tetranychus urticae* Koch (Arac.: Tetranychidae), is an economic pest worldwide including Turkey, causing serious damage to vegetables, flowers, and fruit crops. In recent years, broad-spectrum insecticides/miticides have been used to control this pest in Turkey. Control is difficult mainly due to resistance to conventional pesticides. This study was conducted to determine efficacy of pesticides extracted from five different plants [i.e., *Allium sativum* L. (Amaryllidaceae), *Rhododendron luteum* S. (Ericaceae), *Helichrysum arenarium* L. (Asteraceae), *Veratrum album* L. (Liliaceae), and *Tanacetum parthenium* L. (Asteraceae)] against this mite. Bioassays were tested by two different methods to determine the effects of varying concentrations. Experiments were performed using 3 cm diameter leaf disk from unsprayed bean plants (*Phaseolus vulgaris* L.). In addition, the effects of the extracts on reproduction and oviposition were investigated. The extract yielded high mortality. In the lowest-concentration bioassays, the adult mites laid lower numbers of eggs compared to the untreated control. No ovicidal effect was observed.

1. Introduction

Diseases and insect pests are the major limiting factors in the production of high quality agricultural products. Although conventional pesticides have become an indispensable tool in controlling some pests economically, rapidly, and effectively, extensive use of insecticides may lead to a number of undesirable side effects including the development of insect resistance and resurgence of primary and secondary pests outbreaks. Also they can have adverse effects on nontarget organisms and general environmental contamination [1–4]. The other problems with synthetic insecticides are environmental pollution and insect resistance. According to Nas [5] interest in the application of botanical pesticides for crop protection is on the rise. Many researchers are experimenting and developing alternative plant extracts as pesticides to be used against pest insects.

Plants have the richest source of renewable natural pesticides. Specifically, plant extracts provide a safe and viable alternative to synthetic pesticides and are compatible with the use of beneficial organisms, pest-resistant plants, and to preserving a healthy environment in an effort to decrease reliance on synthetic pesticides. There are many benefits of using botanical pesticides such as reduced environmental degradation, increased safety for farm workers, increased food safety, reduction in pesticide resistance, and improved profitability of production.

As a result, many plant compounds, the majority of which are alkaloids and terpenoids, have now been known to affect insects' behaviour, growth and development, reproduction, and survival [6–9]. Many investigations have recently been performed in relation to effects of plants such as *Chrysanthemum roseum* Web. and Mohr. (Compositae), *Nicotiana tabaccum* L. (Solanaceae), *Derris elliptica* Benth (Fabaceae), neem tree, *Azadirachta indica* A. Juss (Meliaceae), *Melia azaderach* L. (Meliaceae), and *Xanthium strumarium* L. (Solanaceae) on insects [10–13]. The seed kernel extract of neem, known as azadirachtin, has been most

thoroughly tested, and it has been extracted in larger quantities than the other components of neem [14, 15]. High rates of mortality have been found on the two spotted mites fed on the leaves treated with *A. indica* extract. In addition, the same extract significantly reduced the reproductive capacity of mites and the survival of the progeny of treated females greatly diminished in comparison to the control [16].

T. urticae is a very important pest worldwide, causing serious damage to vegetables, flowers, and fruit crops. Many crops must be protected with synthetic acaricides during hot and dry seasons that favor severe outbreaks of *T. urticae*. It is able to transmit many of plants viruses [17].

R. luteum and *V. album* are poisonous plants. It is recorded that the extract of *V. album* has been used as insecticide or rodenticide since the Roman times. Also, today, plants containing toxic alkaloid are used successfully as insecticides and fungicides [18]. In one of the studies evaluating the effectiveness of plant extracts against house flies as indicated, *V. album* inhibited the development of the larvae and the high toxicity [19].

H. arenarium, T. parthenium, and *A. sativum* are important medicinal plants. *H. arenarium*, an infusion of the bright yellow flowers, is used in the treatment of gallbladder disorders and as a diuretic in treating rheumatism and cystitis. It is a component in *zahraa*, an herbal tea used for medicinal purposes in some countries [20]. *A. sativum* and *T. parthenium* have a broad spectrum of biological activity. They have been used for anti-inflammatory, antibacterial, and antifungal activities [21]. It is determined that the extract of *T. vulgare* inhibited the development of *Dermanyssus gallinae* (Mesostigmata: Dermanyssidae). In addition, the same plant extract cultivated showed that it is effective on *T. urticae* [22]. The extract of garlic leaves caused high mortality and reduced reproductive capacity on *T. urticae* [23]. According to the literature, no Works have been published on the acaricidal activity of *H. arenarium*. This study was undertaken in the laboratory at the Central Plant Protection Research Institute in 2009, and the miticidal effect of five plant extracts on *T. urticae* was tested.

2. Material and Methods

2.1. Plants and Preparation of Extracts. This study covered five plant species; *R. luteum*, *H. arenarium*, *A. sativum*, *V. Album,* and *T. parthenium* were tested as an alternative miticidal. Their leaves and stems were collected when plants were at the flowering stage during the years 2008 and 2009. Only the fruit garlic plant was used for this purpose. Ethanol was used as a solvent to extract the required material from five plants for use as an acaricide. The method of Brauer and Devkota [24] was used in preparation of five plants' ethanolic extract.

The materials were stored in the laboratory to dry up. The dried materials were grounded using a blender, and ethanol was added to the dried powder for 72 hours. This mixture was extracted in 5-6 hours using a Soxhlet machine. The ethanol was removed from the extract in a rotary evaporator (50–60°C). For each plant sample 200 g of dried materials were used to prepare the extract.

2.2. Mites. As a test organism, *T. urticae* was reared on green bean plants, *Phaseolus vulgaris*. The bean plants used in the experiment were grown in a greenhouse.

2.3. Effects of the Extracts of Five Plants on Tetranychus urticae. In all the experiments, first instar larvae and 3-day-old adults were used. Four concentrations and an untreated control were used for all bioassays. Test samples for bioassay were resuspended in distilated water with TritonX.100 at a rate of 0.1 mL/L. Vaseline was used so as to prevent the mites from escaping. Experiments were carried out using (3 cm diameter) leaf discs of green bean leaves. The leaf disks were placed on a moistened filter paper disk and each disk was infested with 10 individuals. Each treatment was replicated 10 times. The concentrations used for mites were 1%, 3%, 6%, and 12% [16].

2.4. Effect on Eggs. Green bean leaf discs were placed into petri dishes on moistened filter paper and females of the same age were put on leaf discs. The eggs were counted after two days. Ten eggs were placed in every petri dish and the other eggs removed. Then the eggs were sprayed with different concentrations of extract (17–20 μL/cm^2) using a small hand-held sprayer. The numbers of hatched larvae were recorded.

2.5. Effect of the Extracts on Larvae and Adults

2.5.1. Leaf-Dipping Method. Green bean leaf discs were treated by dipping them into extract solutions of known concentrations, then left to dry for 30 minutes. The treated leaf discs and individual mites were placed in the petri dishes (9 cm in diameter) that were lined with moistened filter paper. The results were assayed after 1, 3, and 6 days by counting the number of living adults and larvae.

2.5.2. Leaf-Spraying Method. Green bean leaf discs were placed into Petri dishes on moisturized filter paper. Ten adults were placed in every Petri dish. Then eggs were sprayed with different concentrations of extract (17–20 μL/cm^2) using a small hand-held sprayer. The results were assayed after 1, 3, and 6 days by counting the number of living adults.

2.6. Effect on Egg-Laying Capacity. Green bean leaf discs were dipped for 3–5 seconds in prepared concentrations (1, 3, 6, and 12%), then they were dried for 30 minutes and placed in petri dishes with ten adults. After 48 hours of feeding on treated green bean leaves, mites were given untreated green bean leaves. The experiment was repeated 10 times. Daily monitoring was done for fourteen days and the total number of eggs was recorded [25].

The experiments were conducted in a climate chamber at 25-26°C and under long daylight (18 h : 6 h, light : dark). The effect was calculated according to Abbott [26]. The obtained reasults were submitted to a variance analysis and the mean values were compared by Duncan's test ($P = 0.05$) calculated by the program SPSS 13.6). Mortality rate was calculated as; mortality = after treatment the number of died mites/before treatment the number of mites · 100).

Table 1: Effect (mean ± SE) and mortality (%) of extracts obtained from different five plants on *T. urticae*.

Treatment		Leaf-dipping method				Leaf-spraying method	
		Larvae		Adult		Adult	
	Concentration (%)	Mortality (%)	Effect (%)	Mortality (%)	Effect (%)	Mortality (%)	Effect (%)
H. arenarium	1	46	31.59 ± 4.00^{c}	37	25.32 ± 4.10^{c}	52	39.76 ± 5.18^{c}
	3	53	41.59 ± 5.47^{bc}	47	37.22 ± 6.77^{bc}	66	59.88 ± 4.65^{b}
	6	58	46.09 ± 2.53^{b}	51	42.36 ± 5.61^{b}	76	71.82 ± 1.76^{ab}
	12	71	62.72 ± 2.28^{a}	64	56.85 ± 5.63^{a}	85	82.38 ± 1.92^{a}
A. sativum	1	46	31.30 ± 5.01^{b}	29	16.43 ± 2.43^{b}	66	59.76 ± 4.45^{b}
	3	50	37.80 ± 5.96^{b}	34	27.59 ± 5.17^{ab}	69	65.45 ± 5.16^{ab}
	6	56	43.37 ± 5.95^{b}	45	34.35 ± 6.76^{a}	77	72.79 ± 4.38^{a}
	12	68	58.35 ± 6.31^{a}	49	39.49 ± 5.07^{a}	78	73.92 ± 3.16^{a}
V. album	1	50	35.83 ± 4.33^{c}	51	29.07 ± 4.71^{c}	47	33.57 ± 4.12^{c}
	3	65	54.93 ± 5.22^{b}	61	42.41 ± 6.33^{b}	59	49.72 ± 3.39^{b}
	6	75	65.37 ± 3.15^{ab}	78	51.57 ± 5.37^{a}	70	62.58 ± 2.98^{b}
	12	77	70.55 ± 2.44^{a}	79	79.02 ± 3.76^{a}	81	75.77 ± 3.81^{a}
T. parthenium	1	49	38.22 ± 5.83^{c}	64	54.49 ± 4.34^{c}	47	33.61 ± 4.14^{c}
	3	64	54.06 ± 3.14^{b}	77	69.58 ± 1.52^{b}	60	49.75 ± 3.41^{b}
	6	75	67.89 ± 2.56^{a}	85	82.41 ± 1.94^{a}	71	62.62 ± 2.96^{b}
	12	82	76.54 ± 3.51^{a}	88	83.47 ± 1.95^{a}	81	75.68 ± 3.77^{a}
R. luteum	1	44	31.87 ± 3.31^{b}	27	31.66 ± 4.50^{b}	37	25.81 ± 2.94^{c}
	3	48	35.38 ± 4.05^{b}	44	34.02 ± 3.62^{b}	42	43.23 ± 3.40^{b}
	6	74	66.14 ± 4.50^{a}	53	44.35 ± 4.43^{b}	58	50.31 ± 3.28^{ab}
	12	81	75.62 ± 3.03^{a}	67	63.66 ± 2.44^{a}	68	61.97 ± 3.75^{a}
Control		22	0	15	0	15	0

Within columns, means ± SE followed by the same letter are not significantly different (DUNCAN's multiple F-test $P < 0.05$).

3. Results and Discussion

3.1. Effect on Eggs. All of the eggs treated were found to have hatched. It is determined that the ethanolic extracts of *R. luteum, H. arenarium, A. sativum*, *V. album*, and *T. parthenium* did not have an ovicidal effect. The hatched larvae continued to develop as it was in the control.

3.2. Effect of the Extracts on Larvae

3.2.1. Leaf-Dipping Methods. From Table 1, it can be observed that ethanol extracts of five plants had a significant mortality and the highest effect on *T. urticae* larvae. In all of the plant extracts, the highest effect occurred at a concentration of 12% while the smallest effect was at 1%. The increased concentration led to increased larval mortality. Statistical analysis showed $P < 0.05$ importance between the treatments. The extract of *T. parthenium* showed the highest effect on the *T. urticae* larvae. The smallest effect was at the extract of *A. Sativum*.

3.3. Effect of the Extracts on Adult

3.3.1. Leaf-Dipping Methods. As shown in Table 1, for the adults placed on leaf discs treated with different plant of extracts, the highest effect was determined at a concentration of 12% the extract of *T. parthenium*. Among the plant extracts, the extract of *T. parthenium* indicated the highest mortality. On the other hand, the smallest mortality was found at the extract of *A. sativum*. The increased concentration led to increased adult mortality.

3.3.2. Leaf Spraying Method. For the larvae placed on leaf discs treated with different plant of extracts at concentration of %12, mortality at the extract of *H. arenarium, A. sativum, V. album, T. parthenium*, and *R. luteum* was 85, 78, 81, 81, and 68%, respectively. In all of the extracts the highest effect was determined at a concentration of 12% while the smallest effect was at 1% (Table 1).

In both methods, similar results were obtained and there was not a significant difference on the mortality when leaf-dipping method was compared with direct spraying on the plant.

3.4. Effect on Egg-Laying Capacity. The numbers of eggs laid by mites feeding on extract-treated bean leaves were found to be statistically significant ($P < 0.05$) for all extracts with the maximum number of eggs obtained from the control. The lowest number of eggs was found at the 12% concentration of the extract of *R. luteum,* and the number of eggs laid was reduced significantly by increasing concentration (Table 2).

Ethanolic extracts were made from different plants and their effects were tested on two-spotted mite for the first time

Table 2: Effect of extracts from obteined different five plants on egg laying capacity of *T. urticae*.

Concentrations (%)	Treatment				
	H. arenarium	*A. sativum*	*V. album*	*T. parthenium*	*R. luteum*
	Number of eggs (mean ± SE)				
Control	162.5 ± 11.80^{c}	162.5 ± 11.80^{c}	162.5 ± 11.80^{c}	162.5 ± 11.80	162.5 ± 11.80^{c}
1	145.5 ± 5.91^{c}	184.0 ± 12.10^{b}	152.6 ± 10.50^{c}	158.0 ± 12.1^{b}	152.6 ± 10.50^{c}
3	94.5 ± 6.0^{b}	154.3 ± 10.3^{b}	137.6 ± 13.43^{c}	153.3 ± 10.3^{b}	137.6 ± 13.40^{c}
6	81.8 ± 6.40^{b}	115.2 ± 9.13^{a}	108.9 ± 19.9^{b}	136.2 ± 9.12^{b}	88.9 ± 19.92^{b}
12	62.5 ± 6.33^{ab}	98.2 ± 8.60^{a}	96.4 ± 2.52^{b}	87.2 ± 8.60^{a}	18.4 ± 2.50^{a}

Within columns, means ± SE followed by the same letter are not significantly different (DUNCAN's multiple F-test $P < 0.05$).

in the world. It was observed that some extracts showed a high rate of mortality and reduced fecundity on *T. urticae.*

There were no references in the literature of other studies using four plant extracts ethanolic extract on *T. urticae* except that *A. sativum*. However, other plant extracts have been investigated and the findings for *T. urticae* are similar to those of our study. Neem seed kernel extracts and its formulation are reported to influence mortality, repellency, and fecundity of mites [27–29]. It was found out that the two commercial preparations of neem seed extracts (Margosan-0 and Neem Azal S, Neem Azal T/S) were effective on *T. urticae* [16, 30]. Several herbal extracts of *Achillea millefolium* L. (Asteraceae), *Taraxacum officinale*s F. H. (Asteraceae), *Matricaria chamomilla* L. (Asteraceae), and *Salvia officinalis* L. (Lamiaceae) demonstrated strong inhibition of the feeding activity of mites [31, 32]. It was determined that the extracts of yew showed a high mortality, decrease in female fecundity and shortened longevity [33, 34]. Shi et al. [35] revealed that the extract of *Bassia scoparia* (L.) A. J. Scott. (Chenopadiaceae) showed contact and systemic effects, and it caused high rates of mortality in all the three species (*T. urticae, T. cinnabarinus*, and *T. viennensis*). Pure azadirachtin reduced the reproductive capacity and feeding of *T. urticae* [36]. Crude foliar extracts of 67 species from six subfamilies of Australian Lamiaceae showed both contact and systemic toxicity to these mites [37]. The extracts of wild tomato leaf showed strong repellency effect on *T. urticae* [38]. The acaricidal activities of plant extracts on *T. urticae* were tested. The mortalities were high in extracts *Albizia coreana* Twig., *Pyracantha angustifolia* F. (Rosaceae), and *Ligustrum japonicum* Thunb. (Oleaceae) within 48 h treatment [39]. Attia et al. [23] revealed that the extract of garlic led to a rise in female mortality and a reduction in fecundity with the increasing of concentration. Essential oils of *Artemisia absinthium* L. (Asteraceae) and *Tanacetum vulgare* L. (Asteraceae) were extracted by three methods, a microwave-assisted process (MAP), distillation in water (DW), and direct steam distillation (DSD), and tested for their toxicity as contact acaricides to *T. urticae.* DSD and DW extracts of *T. vulgare* were more toxic (75.6 and 60.4% mite mortality, resp., at 4% concentration) to *T. urtica* than to the MAP extract (16.7% mite mortality at 4% concentration) [22]. The ethanol extracts *of Croton rhamnifolius* H.B.K. (Euphorbiaceae) *C. sellowi, C. jacobinensis, and C. micans* had a high mortality on *T. urticae,* whereas *C. sellowi* extract showed the highest effect [40]. Garlic extract showed a mortality at 48–57% on *T. urticae* [41]. Wang et al. [42] revealed that the crude extract of walnut leaf had some contact and systemic effect on *T. cinnabarinus* and *T. viennensis*.

It was found out that the extract of *V. album* and *T. parthenium* had a high rate mortality and reduced fecundity for *T. urticae.* Ethanolic extracts of *V. album* and *T. parthenium* can be useful to control *T. urticae* populations on vegetable plants grown through Integrated Pest Management (IPM) and organic systems of agriculture.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 481509, 23 pages
doi:10.1155/2012/481509

Research Article

Butterfly Diversity from Farmlands of Central Uganda

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Received 9 October 2011; Accepted 31 May 2012

Academic Editor: Robert Matthews

The aim of this study was to collect information about the diversity of butterfly communities in the mixed coffee-banana mosaic (seminatural, agricultural) landscapes of rural central Uganda. Data were collected for one year (2006) using fruit-bait traps, line transect walk-and-counts, and hand nets. A total of 56,315 individuals belonging to 331 species, 95 genera, and 6 families were sampled. The most abundant species was *Bicyclus safitza* (14.5%) followed by *Acraea acerata* (6.3%), *Catopsilia florella* (6.5%) and *Junonia sophia* (6.1%). Significant differences in abundance, species richness, and diversity of butterflies occurred between the 26 study sites. Farmland butterflies visited a variety of habitats within and around sites, but important habitats included woodlands, fallows, hedgerows, swampy habitats, abandoned gardens, and home gardens. The highest diversity and abundance of butterflies occurred in sites that contained forest remnants. Thus, forest reserves in the surrounding of fields increased the conservation values of coffee-banana agroforestry systems for butterflies. Their protection from degradation should be a priority for policy makers since they support a species-rich community of butterflies pollinating cultivated plants. Farmers are encouraged to protect and increase on-farm areas covered by complex traditional agroforests, linear, and nonlinear seminatural habitats to provide sufficient breeding sites and nectar resources for butterflies.

1. Introduction

Butterflies are considered as good ecological indicators for other invertebrate taxa [1–3] and as surrogate representatives of environmental quality changes. Butterflies play significant ecological roles in agricultural landscapes. They perform essential ecosystem services [4], especially in the recycling of nutrients (N, P, K) highly needed by crops that were previously taken through plant absorption and uptake. Their larval stages feed on leaves of several wild plants found in the agricultural systems and therefore release their faeces that contain some amount of nutrients [5]. In addition, butterflies are food to birds and other predators and are hosts to several parasitoids that suppress crop pests [6].

Despite their diversity, ubiquity, and importance particularly with regard to their ecology, behavior, and functional role (e.g., pollination activities), butterflies remain relatively little studied in farmland habitats [5] in the tropics. In agricultural systems, several butterfly species are suspected to be important pollinators of wild and cultivated crop species on which human beings depend on for their livelihoods [7].

Among the invertebrates, butterflies are one of the best studied insect groups for which both ecological and relatively good quantitative distribution data are available worldwide. In Mediterranean countries [8], knowledge of butterflies inhabiting farmland habitats is fairly good compared to most of the regions of sub-Sahara Africa where the knowledge is poor or absent.

Roughly 90% of butterfly species live in the tropics [7]. Despite this, very little is known about tropical farmland butterfly ecology particularly when compared to temperate butterfly systems. The relative scarcity of data on

tropical butterfly species hampers the ability to effectively conserve them, particularly as pollinating agents in agricultural systems.

In Uganda, most studies on butterflies have been carried out in natural areas, forest ecosystems, and in protected areas [9–12]. There is a lack of empirical data on butterflies from farmland habitats in sub-Sahara Africa and in Uganda. No published data exists describing the diversity of butterflies found in agricultural landscapes in Uganda. However, such information is important for butterfly biodiversity and ecosystem services conservation on farmlands.

Because butterflies provide important ecological services for crops and native wild plant species in many ecosystems of the world [13], their conservation is essential to sustain the productivity of natural and agricultural landscapes. However, the protection, conservation, and utilization of Lepidoptera pollinator diversity require extensive understanding of their foraging behaviours and of their temporal and spatial distribution in agricultural landscapes [14, 15]. Farmland habitats support a rich and functionally diverse butterfly community.

Preliminary field observations conducted in central Uganda [7] indicated that some butterfly species are present and busy collecting floral resources in vegetable and legume crop fields later in the morning hours to evening time. Although a proper assessment of pollination efficiency of different species has not been conducted, the abundance and long visits to crop flowers indicated that some of these crops were receiving butterfly pollination services [7]. Preliminary observations indicated also that the annual contribution of butterflies to total pollination services delivered to crops in Uganda is in the range from 4.0% to 9.0% [7]. Thus, butterflies may constitute the second group of crop pollinators behind bees in central Uganda [7]. It is likely that some species are both good pollinators of some cultivated and wild plant species. There is a need to conserve butterflies in rural landscapes of central Uganda.

The aim of this study was to collect information on the current status of butterflies in farmlands of central Uganda. The specific objectives were (i) to characterize butterfly assemblages (abundance, species richness and diversity) on farmlands of central Uganda, (ii) to compare butterfly assemblages in sites with different seminatural habitats/land-uses and farm management systems, and to identify environmental factors (land-uses, habitats) mostly determining the distribution and occurrence of different species in the farmlands of central Uganda.

In this study, it was hypothesized that farmlands do not support diverse butterfly communities. It is predicted that butterfly assemblages on farmlands in sites with different land-uses, seminatural habitats and farm management systems in central Uganda are of similar taxa given the farmland character that is over-cultivated.

2. Material and Methods

2.1. Study Area. This study was conducted in the banana-coffee system of Lake Victoria Arc covering several districts of the central Uganda (Figure 1). The study zone is characterized by ferrisoils with high to medium fertility level and receives on average 1000–1800 mm of rains on a bimodal pattern (rainy seasons: March–May, September-November; semi-dry to dry seasons: June-August, December-February) with 22–28°C and 60–75% of mean annual temperature and relative humidity respectively [7]. The study zone is farmland area found around Lake Victoria. The farmland zone is characterized with shrubs of *Acacia* spp., legume trees, melliferous plant species, *Papyrus* and palms ranging from 2 to 15 m high dominating the remnant secondary vegetation.

Several food and cash crops are grown, mainly cassava (*Manihot esculentum* L.), sweet potato, (*Ipomoea batatas* L.), maize (*Zea mays*), beans (*Phaseolus vulgaris* L.), groundnut (*Arachis hypogea* L.), tomato (*Lycopersicon esculentum*), watermelon (*Citrullus lanatus*), pumpkin (*Cucurbita moschata*), cucumber (*Cucumis sativus*), melon (*Cucumis melo*), chilies (*Capsicum* spp.), and several other fruits, vegetables and horticultural crops (cabbage, onion, egg plants, sim-sim, etc.). The majority of these crops are grown in small-scale monoculture and/or polyculture fields that are integrated into the coffee-banana agroforest production systems where coffee and banana are the major crops.

Rural central Uganda is mosaic landscape where "islands" of patches of natural habitats (forest reserves, wetlands, woodlands) are found scattered within agricultural matrices dominated by linear and nonlinear features of seminatural habitats (fallows, hedgerows, grasslands, woodlots, rangelands) that serve as field boundaries for a diversity of small-scale fields. Compared to other regions of Uganda, central region is characterized by high demographic pressure, limited access to arable lands, continuous cultivation, and overexploited lands.

This study was conducted at 26 study sites that were previously selected to represent a range of land-use types of varying degrees of anthropogenic disturbances and farm management intensities. Thus, all study sites were characterized by the presence of contrasting habitat and land-use types in the vicinity. The different study sites were grouped into clusters such as each cluster contained 2 to 4 study sites. The different study sites were separated from one another by a distance from 5 to 50 km. The different study sites were later regrouped into different farm management systems (modernized large-scale fields, traditional small-scaled fields). They were also grouped into clusters of different land-use intensity gradients or categories (low, medium, high, very high). Detailed description of the different land-use intensity classes occurring in the study area are presented in Munyuli [7]. Characteristics of dominant agroforestry systems found in central Uganda are presented in Table 1.

2.2. Field Sampling of Butterflies. Field sampling of butterflies was conducted at 26 study sites. In each of the 26 study sites, an area of 1 km^2 was selected and the area was divided into five linear parallel transects of 1000 m long and 200 m apart (Figure 2). Transects were used as basic sampling

Table 1: Type of common agroforestry systems found in farmlands of central Uganda.

Cluster names	Study site names	Type of traditional agroforestry systems	Size range (ha) of the agroforest	Other vegetation characteristics of the traditional agroforests	Closeness or distance to water bodies	Dominant natural habitats, seminatural habitats, and vegetation types found within, around, and in the vicinity of sites
Bujaggali	Bukose	Shaded coffee-banana agroforests	10–100	Mass blooming of weeds/herbs in the ground layer	30 m to wetlands	Reclaimed wetlands, young fallows, field margins, woodlands
	Namizi-east	Multistrata complex shaded banana-coffee agroforests	10–500	(i) Multipurpose agroforest tree species (ii) Forest remanent tree species	4000 m to swamps	Forest fallows, young fallows, grasslands, pastures, marshlands, swamps, wetlands
	Namizi-west	Simple banana-coffee agroforests	10–50	(i) Tin wild woody trees in the lower layer (ii) Some invasive tree species	5000 m to river Nile	Conifer plantations, pine/eucalyptus plantations, old fallows, grazing fields, large swamps, forest fallows
	Nawangoma	Simple agroforests, homegardens	10–100	(i) Diverse shade tree species (ii) Diverse fruit tree species (iii) Ruderal vegetation in bloom	7000 m to Lake Kyoga	Woodlands, shrublands with several deciduous trees, hedgerows, field margins, forest plantations, degraded/cleared forest patches
Kalagi	Bamusuta	Complex shaded banana-coffee agroforests	10–50	(i) Mass blooming weeds/herbs in ground layer	1000 m to wetlands	Young fallows, hedgerows, forest fallows
	Kifu	Complex banana-coffee agroforests	10–700	(i) Mass blooming of ruderal vegetation in the ground layer, (ii) Native tree species in the canopy	200 m to swamps	Wood-shrub grasslands, grasslands, cattle pastures, swampy forests, forest reserves
	Kimwanyi	Simple shaded coffee-banana agroforests	10–50	(i) Multistrata mass blooming weeds/herbs at the ground layer (ii) Coffee shrubs in the upper layer	500 m to streams	Eucalyptus plantations, wood-shrub grasslands, young fallows, hedgerows, cattle pastures, forest plantations, large wetlands
	Kiweebwa	Complex banana coffee-agroforests	10–900	(i) Mass blooming of weeds/herbs/grasses in the ground layer (ii) Coffee shrubs in the upper layer	600 m to swamps	Forest fallows, swampy forests, woody grasslands, woodlots, Invasive tree species, young fallows, hedgerows
Kamuli	Naikesa	Simple agroforests	10–20	(i) Mass blooming of ruderal vegetations (ii) Coffee shrubs in the upper layer	1600 to swamps	Forest fallows, old fallows, grazing fields, shrublands, swamps
	Namulekya	Multistrata complex agroforests	10–200	(i) Tin woody trees (ii) Tall herbs in lower layer (iii) Coffee shrubs in the upper layer (iv) Diverse indigenous shading trees	400 m to swamps	Swampy forests, eucalyptus plantations,young fallows, pine plantations

Table 1: Continued.

Cluster names	Study site names	Type of traditional agroforestry systems	Size range (ha) of the agroforest	Other vegetation characteristics of the traditional agroforests	Closeness or distance to water bodies	Dominant natural habitats, seminatural habitats, and vegetation types found within, around, and in the vicinity of sites
Kaweri	Luwunga	Large shaded coffee plantations	10–60	(i) Diverse shade tree species (ii) Mass blooming weeds/herbs in the ground layer	200 m to river channels	(i) Forest fragments in the vicinity of the coffee plantations, (ii) Presence of rivers and irrigation channels
	Nonve	Large sun coffee plantations	10–900	(i) Simple and scattered presence of native plant species in the coffee plantations	500 m to rivers	(i) Forest fragments in the vicinity of the coffee (ii) Presence of rivers and irrigation channels
Lugazi	Kasaku tea	Large sun tea plantations	50–750	(i) Mass blooming of weeds/herbs in the ground layer (ii) Tea shading trees	200 m to Swamps	Wood-shrub grasslands, swamps
	Sugar	Large sun sugar cane plantations	5–10	(i) Mass blooming of weeds/herbs in the ground layer	200 m to irrigation channels	Shrublands, irrigation channels
Mabira	Bulyasi	Complex forest-agroforest systems	10–400	(i) Mass blooming weeds/herbs (ii) Coffee shrubs, tall herbs, (iii) Indigenous	2000 m to streams	Eucalyptus plantations, forest fallows, young fallows, grazing fields, mabira large forest reserves
	Kinoni	Simple agroforests, homegardens	10–760	(i) Native medicinal plant species, (ii) Diverse shade trees (iii) Diverse fruit tree species (iv) Multistrata mass blooming weeds/herbs/grasses	8000 m to marshlands	Forest fallows, young fallows, old fallows, grazing fields
Masaka	Kasaala	Simple agroforests	10–100	(i) Coffee shrubs in the upper layer (ii) Simple shad tree species	12000 m to rivers	Young fallows, old fallows
	Katwadde	Simple agroforests	10–100	(i) Diverse tree species (ii) Presence of forest remnant trees	17000 m to rivers	Young fallows, hedgerows, grazing plots
	Kiwaala	Complex coffee-banana agroforests	10–980	(i) Multiple native shade tree stands (ii) Multistrata of blooming of weeds/herbs/grasses (iii) Forest remnant trees	12000 m to wetlands	Ecotones, forest fallows, old fallows, hedgerows, cattle pastures, woodlands, forest reserves, wetlands
	Mpugwe	Simple agroforests	10–200	(i) Dense native shade trees (ii) Mass blooming of weeds/herbs	18000 m to rivers	Forest remnants, young fallows, old fallows, grasslands, shrublands

Table 1: Continued.

Cluster names	Study site names	Type of traditional agroforestry systems	Size range (ha) of the agroforest	Other vegetation characteristics of the traditional agroforests	Closeness or distance to water bodies	Dominant natural habitats, seminatural habitats, and vegetation types found within, around, and in the vicinity of sites
Mpigi	Lukalu	Complex agroforests	10–600	(i) Mass blooming weeds/herbs in the ground layer	200 m to streams	Young fallows, cattle pastures, hedgerows, forest fallows, intact natural forest reserves
	Mpanga	Complex traditional agroforests	1100	(i) Mixture of coffee shrubs, tin woody trees, tall herbs, diverse fruit species	300 m to wetlands	Swamps, forest wetlands, young fallows, grazing fields, forest fallows, shrublands, forest reserves
Nakaseke	Kimuli	Multistrata complex agroforests	10–400	(i) Mass blooming weeds/herbs (ii) Dense native trees in the canopy layer	200 m to large wetlands	Ecotones, forest fallows, cattle pastures, grasslands, cleared forests, forest fallows, secondary forests, swamps, large wetlands
	Kyetume	Complex agroforests	10–400	(i) Mass blooming weeds/herbs (ii) Dense native trees in the canopy (iii) "Semi-forests" of coffee trees	2200 m to large wetlands	Woodlands, shade tree stands, young fallows, cattle pastures, woodlands, primary forests, secondary forests
	Lukumbi	Complex agroforests	10–200	(i) Mass blooming weeds/herbs (ii) Dense native trees in the canopy (iii) Forest remnant trees	3800 m to large swamps	Woodlands, young fallows, grazing fields, forest fallows, secondary forests, SEMUTO large wetlands
	Segalye	Complex agroforests	10–600	(i) Mass blooming weeds/herbs (ii) Dense native trees in the canopy (iii) Forest remnant trees	4890 m to large swamps	Old fallows, woodlands, degraded secondary forests, large woodlands

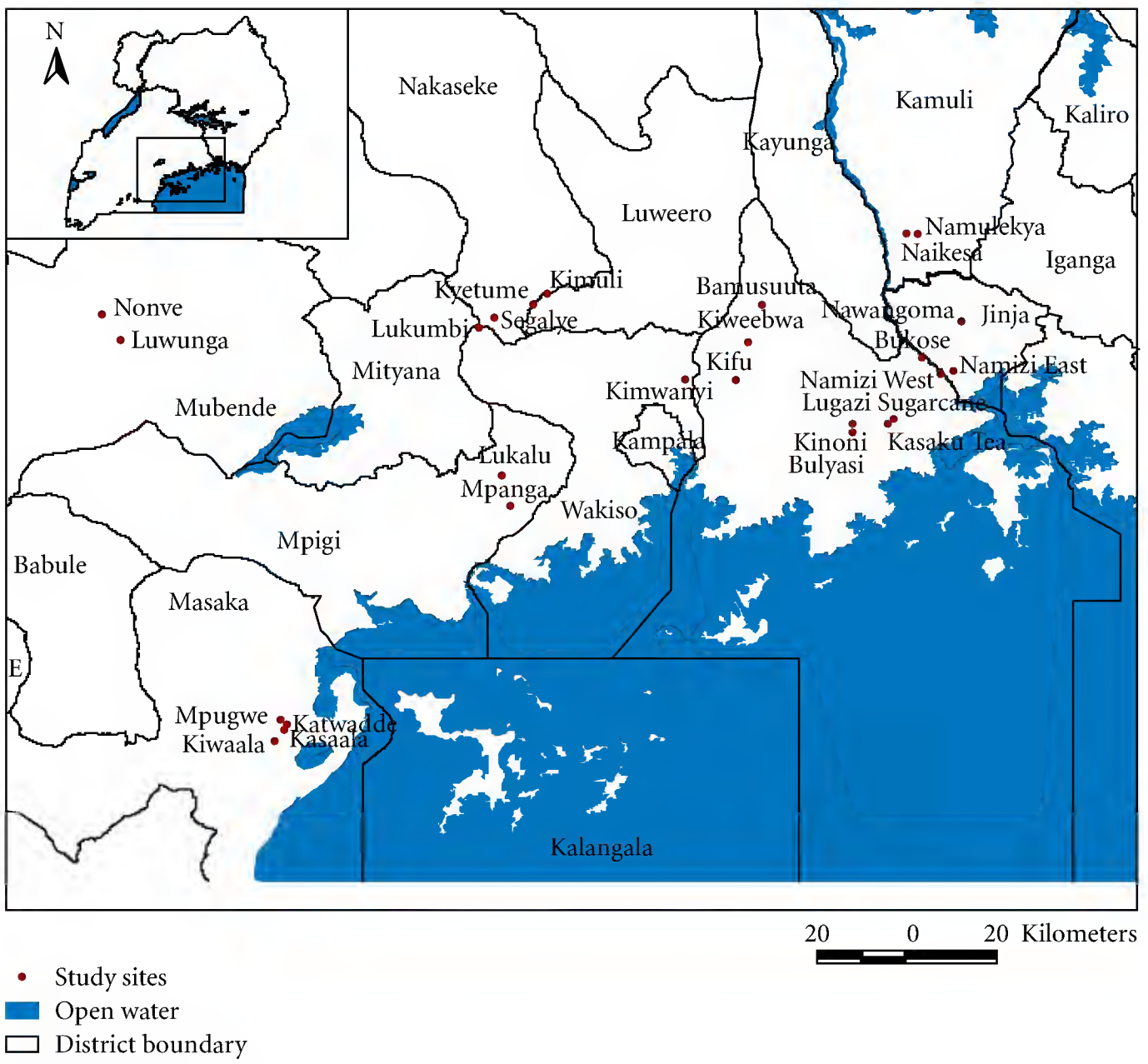

FIGURE 1: Location of districts and study sites from the banana-coffee farming system located around Lake Victoria in Uganda.

unit for all butterflies, land-uses, seminatural habitats, and vegetation data. Thus, butterflies were sampled on one central transect per study site due to time and resource constraints and due to the high number of sites targeted to be sampled during every data collection round. The central transect was selected to represent different seminatural habitats, vegetation, and land-use types of transects that were not sampled. The selected transect was surveyed for five rounds of data collection. A "sampling belt" (20 m wide × 1000 m long = 2 ha) or "sampling plot" was selected in the middle of each central transect [7]. The size of the sampling plot was fixed based on author field experience. The "sampling plot" was kept constant across all sampling dates conducted in each study site.

Each sampling period, the sampling was conducted by two surveyors (the author and one research assistant). To reduce on biases and obtain confidential data, the research assistant was previously trained in butterfly sampling techniques. To assess data reliability, the repeated counts per transect were the same on each sampling date. Using each of the three sampling methods above mentioned, butterflies were sampled during five consecutive rounds from January 2006 to December 2006 (Round 1: January–April, Round 2: May-June, Round 3: July-August, Round 4: September-October, and Round 5: November-December) in each of 26 sites studied. During sampling periods, weather data in the different study sites were similar across rounds. Sampling was conducted across crop growing seasons in order to enable further comparisons of results between rainy (wet) and dry seasons. Butterflies were sampled under good weather conditions during sunny and calm days from 09 h00 to 17 h00.

Overall, butterflies were sampled using three complementary methods (transect walk-and-counts, hand nets, and fruit bait traps) universally recommended and extensively used to survey and monitor butterfly populations and communities [16–18] in the tropics. These methods have been applied in Uganda in previous studies [19–21]. Details of the three sampling methods applied are presented in Munyuli [5]. The different sampling methods are hereby briefly presented.

2.2.1. Transect Walk-and-Counts. Butterfly communities were counted using line transect method also called "visual census method" [7]. During each sampling visit, butterflies were counted while walking at a steady pace of 5–10 m/min along transect lines, frequently stopping, and observing butterfly species within transect range. With experience and ability to identify correctly as many species as possible by observers/recorders, butterflies were identified on the wing (using wing characteristics) while flying out and the total number of butterflies of each species flying within view of the observer was recorded. Butterflies that could

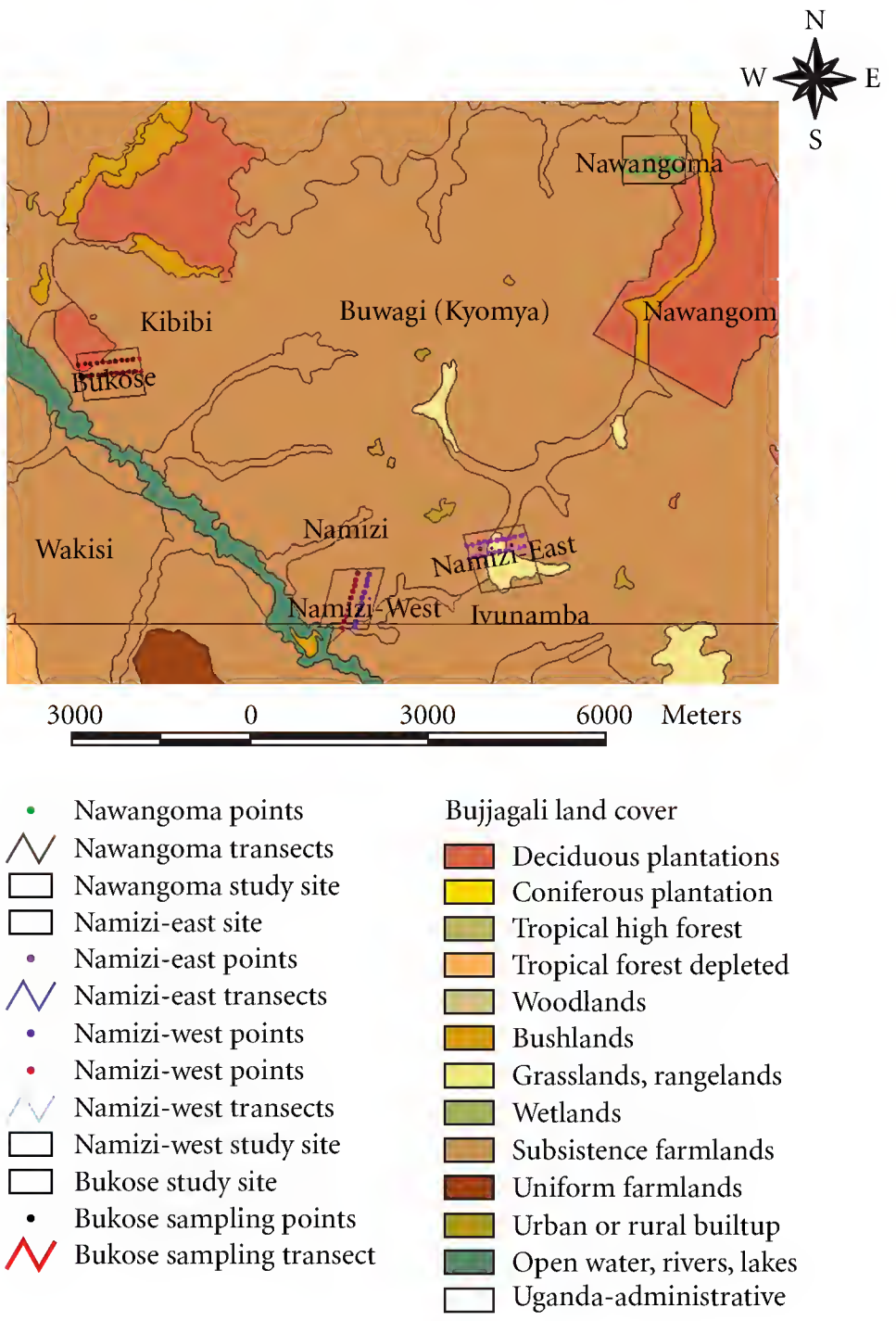

FIGURE 2: Location of Bukose, Namizi-east, Namizi-west, and Nawangona study sites in Jinja district.

not be identified on spot were captured for laboratory identification. While sampling to both sides of the transect, caution was taken to sufficiently avoid double counting or recounting of individuals of a given species by walking in one direction and by not moving back to resample a species seen behind the surveyor. To avoid counting individuals and species more than once, they were released far away from the sampling belt (transect). Thus, during transect walks, specimens were not collected.

2.2.2. Hand-Netting Method. Hand-netting was carried out immediately after visual counts were finished, using an ordinary insect hand net. Hand-netting was conducted for 20 to 30 min per transect and involved sampling ("hand-netting") all butterflies that could not be identified on-spot during visual censuses, at every patch of nectarine plants that could be found alongside the transect. Captured butterflies were counted. Majority of captures were released after field identification, doubtful specimens were collected in paper packets and taken to the laboratory for proper identification.

2.2.3. Banana Fruit-Bait Traps Method. Butterflies were captured in traps baited with mashed ripe bananas that had been fermenting in plastic buckets for 2 days. The bait was prepared three days prior to baiting and not replaced unless lost. Traps were made with local materials, based on the Van-Someron-Rydon trap design [12, 21]. Thus, the traps were cylinders of white nylon netting (125 cm high and 35 cm in diameter) sewn onto a frame of two metal rings, closed at the top and open at the bottom. The trap nets were 125 cm in height, minimizing the risk of escape once butterflies had entered. Traps were placed at about 100 m interval along transect lines. At each trap point, one trap was placed in sunny area while the other was hanged in a shaded habitat to maximize the chance of trapping species attracted by different habitat management in the agricultural landscapes. Each trap baited with two spoons of fermenting banana, was suspended 1.5–2 m above the ground. Traps placed in sunny or shaded environments were hanged on trees and shrub branches using a slingshot and weighted fishing line. Traps were installed preferably in the evenings between 16 h00 and 18 h00 and samples removed after 48 h. Overall, twenty-baited traps were set each sampling visit in each study site. During the untrapping process, butterflies in the traps were counted and few individuals of the same species were collected in glassine paper envelopes for identification in the laboratory at Makerere University, the rest of the specimens found alive were released after they being recorded.

Butterfly specimens were identified by consulting literature, nomenclature, and colored plates of butterflies and by using the reference collection available at Makerere University Zoology Museum. The taxonomic characteristics of butterflies were obtained from standard guides including (i) *Butterflies of Kenya* [22]; (ii) "*Butterflies of West Africa*" [23]; (iii) "*Butterflies of Uganda*" [24]. Identification of all voucher specimens was confirmed by a butterfly taxonomist (Mrs. Akite Perpertra) based at Makerere University Zoology museum. A collection of butterfly voucher specimens from the 26 study sites is deposited at the Zoology Museums, Makerere University. Butterfly collection permit in farmlands of Uganda was issued by the Uganda national council of Science and technology (UNCST). Butterflies were collected under an officially registered project in Uganda (Darwin project reference: 14-032).

2.3. Environmental Factors. A variety of "environmental factors" (land-uses, seminatural habitat, and natural habitats) are used as foraging/breeding habitats by diverse butterfly species in central Uganda. These "environmental factors" are likely influencing occurrence of different foraging butterfly species on transects. Concurrently with butterfly surveys, different land-uses and seminatural habits that met within and in the vicinity of crop fields during transect walks were noted every sampling day in each study site. The size (ha) of the habitat was also recorded simultaneously to the registration of butterfly species and individuals visiting nectarine patches within the habitat/land use.

During transect walks, activities of different butterfly species on blooming crops were noted. In each study site

and in each habitat/land use, the number of plant species in bloom was recorded each sampling day in order to estimate the availability of nectarine resources for butterflies on a monthly basis. In addition, average climatic data (rainfall, temperature) for year 2006 was calculated using raw data from 4 meteorological stations located in the study zone for year 2006. Detailed information on meteorological data from the study region can also be obtained from the office of the prime minister, government of Uganda, meteorological department, Kampala, Uganda [7].

2.4. Butterfly Biodiversity Analysis. For the basic biodiversity analysis, the data from the three sampling methods were pooled to obtain total butterfly biodiversity per 2 ha transect per study site per sampling round as recommended [7]. In addition to personal field observations, data on butterfly functional groups were obtained from published records. The nomenclature of butterfly habitat associations and ecological group was adopted from Larsen [22, 23] and from Davenport [25].Thus, every species recorded was assigned to one of the following ecological habitat categories: F = forest-dependent species; f/m = forest edge/woodland habitats and migratory specialist species; f = forest edge/woodland species; O = open habitat specialist species; O/m = open habitat/migratory species; S = swamp/wetland specialist species; W = widespread species; U = species of unknown (undescribed) habitat preference; W/m = widespread species and migratory species; m = migratory species. The proportion of the F and f species in a sample is an indication of the ecological state (habitat of good quality) of the habitat when working in forest habitats [19] and in similar agricultural matrices. For each ecological category, species richness and abundance were calculated using pooled data from the three sampling methods.

For the biodiversity analysis, species dominance (D) was calculated according to Buschni and Woiski [26]: D = (abundance of a species/total abundances recorded) $\times$ 100. If $D > 5\%$, the species was termed a dominant species; if $2.5\% < D < 5\%$, the species was termed an accessory species or species of intermediate abundance, and if $D < 2.5\%$, the species was termed as an incidental species. Rare species (species represented by less than 5 individuals and or sampled from only one site) and unique species (species occurring with one individual: singleton, or with two individuals: doubleton) were noted whenever present per sample obtained each sampling round as recommended [26, 27].

Species richness was estimated by considering the number of butterfly species recorded each sampling round [27]. Total species recorded per study site was used to get the average since the interest was to detect the pattern. Butterfly faunal diversity was estimated by calculating the Evenness and the Shannon diversity indices as recommended [27].

Species accumulation curves, which plot the cumulative number of species (S) as a function of sampling effort, were used as recommended [27]. To determine the degree to which the sampling effort and protocol accurately reflected the butterfly community, species accumulation curves were generated based on continuous samples obtained in each round of data collection as recommended [27]. Species accumulation and estimation curves were generated using the Jacknife1 estimator. Jacknife-1 was calculated using Microsoft Excel 2003 computer program following approaches described by [27].

The Sorensen similarity index [27, 28] was used to compare similarities among sampling methods. Sørensen's index of similarity (IS) was calculated using the following equation: IS = $[(2C/2C + A + B)] \times 100$, where C is the number of species in common between two treatments having A and B number of species, respectively. An IS value of 100 indicates that the two treatments contain the same number of species (complete overlap among sampling methods), whereas an IS value of 0 indicates that the two treatments have no common species (there is no similarity in community composition between sampling methods).

2.5. Statistical Data Analysis. All variables were tested for normality and the strongly skewed variables were transformed prior to analyses if necessary to meet the assumption of normality and homogeneity of variances. Variables expressed as percentages (%) were arcsine-square-root (+0.5) transformed and number of species or counts of individuals were $\ln(x + 1)$ transformed. However, when log transformation of the raw data was performed and if the data was still skewed, an appropriate nonparametric test was applied. Back-transformed data are reported.

The differences in butterfly individuals and species, evenness and diversity between the 26 study sites were tested with General Linear Model (GLM) analysis of variance (ANOVA) in Minitab release version 15. Where the GLM test indicated significant differences, post-hoc Fishers' least significant difference (LSD) test was used.

To find out whether large-scale fields supported rich and diverse butterfly communities compared to small-scale fields, the General Linear Model (GLM) was applied with Fisher's protected least significant difference (LSD) test for mean separation at 5% probability.

Data on butterfly attraction to different habitat/land-use types found in the 26 study sites was skewed even after transformation. Therefore, to identify best habitat/land-use types for butterflies inhabiting farmlands, a nonparametric test was applied to investigate the effect of habitat types on the richness and abundance of butterflies.

The relationships of foraging butterfly species and "environmental factors" were explored using CANOCO program, version 4.5 [29]. A unimodal direct gradient analysis of partial canonical correspondence analysis (CCA) was used to relate the variation of butterfly communities to environmental variables. Different land-uses and seminatural habitats recorded on transects were put in the data matrix of independent environmental factors, whereas recorded foraging butterfly species were grouped in the data matrix of dependent variables. The ordination analysis was based on all butterfly species that were entered in the model with 10 environmental factors. Environmental variables were selected using manual forward selection. Under a

reduced model for the canonical axes (499 randomizations), Monte Carlo permutation tests were used to assess statistical significance of the association (linear relationship) between butterfly community species composition and environmental factors. Results from the CCA produce eigenvalues that are used to describe how much variance is explained by each ordination axis, thus measuring the importance of each axis. Whenever the significance of the whole ordination was obtained, a forward stepwise method was used to determine which environmental variables were correlated to axes and explained variation in butterfly species composition. A test for significance of correlation of environmental factors with the 4 different axes was conducted using Spearman rank of correlation. To illustrate the relationships between environmental variables and species, biplots of the respective partial CCA were produced.

3. Results

3.1. Butterfly Assemblages. Overall 56315 butterfly individuals belonging 6 families, 15 subfamilies, 24 tribes, 95 genera, and 331 species (Table 2) were sampled across the 26 study sites. There were 120 singleton species, 80 doubleton species, and 59 rare species. The highest estimated species richness was captured by hand nets (where Jacknife-1 revealed a very steep final slope). The species accumulation curve was relatively steep, not reaching an asymptote in hand net and transect counts although beginning to reach a plateau by round four in banana-bait trapping method. Similarly, the overall species accumulation curves for butterflies did not saturate. Because the curve did not saturate, this indicated that not all species of butterfly present in central Uganda were sampled. Overall, the total number of observed species was 331 while Jacknife1 predicted a total of 360 species. This indicates that 91.2% (331 of 360 estimated species) of the species present in the study area during the period of study were sampled.

In this study, the highest species richness was captured in hand nets (Figure 3). Most (75% of total individuals) butterflies were recorded through line transect counts and very relatively individuals were captured by hand net (19%) and banana-bait trap (6%). Hence, hand-net and banana-baits methods sampled statistically ($P > 0.05$) similar butterfly individuals.

There were differences between average similarity index values (Sorensen similarity index) of species shared between the three sampling methods. The percentage of shared species (measured here as % of similarity in species composition) between the sampling methods was highest between hand net and transects counts (41.9%) followed by hand net and banana-bait traps (31.8%) and least between banana-baits and transect counts (21.5%). These results also indicate that use of multiple techniques can considerably increase the number of species.

The abundance of different butterfly taxa varied among sampling methods. Overall, the most abundant species was *Bicyclus safitza* (14.5%) followed by *Acraea acerata* (6.3%), *Catopsilia florella* (6.5%), and *Junonia sophia* (6.1%). Also, the most abundant genus was *Bicyclus* (21.6%) followed by *Acraea* (13%), *Junonia* (6.1%), and *Eurema* (5.7%). For the fruit bait traps, *Bicyclus safitza* (23.3%), followed by *Eurytela drope* (6.4%), were the most abundant species. The most abundant genus trapped using banana-bait trap was *Bicyclus* (45.2%) followed by *Eurytela* (8.3%), *Acraea* (5.9%), *Junonia* (5.7%), and *Neptidopsis* (4.5%).

Most (44%) of the 331 species sampled were "forest-dependent species," followed by widespread species (31.4%) and open habitat specialists (13%) (Table 2). Species belonging to the other ecological trait categories constituted less than 12% of the total species sampled. Forest edge/woodland had the least proportion of species sampled (Table 2).

Variation in butterfly abundance, species richness, and diversity between the 26 study sites within the different sampling methods were striking (Table 3). The highest abundance was associated with Kiweebwa study site whereas the site with lowest abundance was Nonve (Table 3). The highest butterfly species richness was found in Lukalu and the lowest in Nonve study sites (Table 3). The highest diversity index value was found in Kimuli study site and the lowest in Nonve study site (Table 3). There was no clear pattern for the land-use intensity gradient. Study sites located in regions with low land-use intensity were not necessarily species study sites that supported high species richness. For example, the highest specie richness was recorded in Lukalu site that is located in region with medium land-use intensity. However, study sites with very high land use intensity were the least species-rich sites: Nonve and Luwunga (Table 3).

The pattern of butterfly species richness and abundance varied across sampling rounds and sampling methods. Average butterfly species richness ranged from 19 to 32 species across sampling rounds, with the highest richness occurring during the first round and the least in the fifth round of data collection. Butterfly abundance was higher in the first and second sampling rounds than in the rest of the rounds. Overall, butterfly species richness and abundance varied seasonally, with more species and higher densities in the rainy seasons (wet months) compared with the dry season (dry months).

3.2. Environmental Factors Determining Occurrence of Different Species. Butterfly community was significantly related to environmental factors measured (Figure 4; Table 4: CCA; sum of all canonical variables = 1.635; $F = 1.286$, $P = 0.01603$ for all canonical axes). The first, second, third, and fourth axes explain 26.6%, 24.2%, 23.2%, and 20.4% of the variance, respectively, in the model. The correlation of the environmental variables with the axes is summarized in Table 5. Few environmental factors significantly correlated with axes (Table 5). Axis 3 had a high loading for the most determinant factors of butterflies in farmland of central Uganda: small-scale agroforestry fields with fruit tree species, large coffee plantations in the margins of native forest fragments, swampy habitats, reclaimed wetlands, stream-edges, Eucalyptus/Pine plantations, woodlands, grasslands, large tea plantations in vicinity of wetlands/swamps, small-scale monoculture/polyculture fields of annual/biannual crops,

Table 2: Systematic list of butterfly species collected from farmlands of central Uganda in 2006. The species are arranged per family, subfamily, and par ecological category.

Family	Subfamily	Species	E-cat
Riodinidae	Nemobiinae	*Abisara neavei neavei* (Riley)	FDS
Hesperiidae	Hesperiinae	*Acleros mackenii olaus* (Plötz)	FEW
Nymphalidae	Heliconiinae	*Acraea lycoa* (Godart)	N
Nymphalidae	Heliconiinae	*Acraea acerata* (Hewitson)	WSS
Nymphalidae	Heliconiinae	*Acraea acrita* (Hewitson)	N
Nymphalidae	Heliconiinae	*Acraea aganice* (Hewitson)	SWSS
Nymphalidae	Heliconiinae	*Acraea alicia* (Sharpe)	N
Nymphalidae	Heliconiinae	*Acraea asboloplintha* (Karsch)	SWSS
Nymphalidae	Heliconiinae	*Acraea baxteri philos*	FEW
Nymphalidae	Heliconiinae	*Acraea bonasia bonasia*	FDS
Nymphalidae	Heliconiinae	*Acraea boopis ama* (Pierre)	FDS
Nymphalidae	Heliconiinae	*Acraea cabira* (Hopffer)	FEW
Nymphalidae	Heliconiinae	*Acraea egina egina* (Cramer)	WSS
Nymphalidae	Heliconiinae	*Acraea encedon encedon* (Linnaeus)	WSS
Nymphalidae	Heliconiinae	*Acraea eponina eponina* (Cramer)	WSS
Nymphalidae	Heliconiinae	*Acraea jodutta jodutta* (Fabricius)	FDS
Nymphalidae	Heliconiinae	*Acraea johnstoni johnstoni* (Godman)	FEW
Nymphalidae	Heliconiinae	*Acraea leucographa* (Ribbe)	FDS
Nymphalidae	Heliconiinae	*Acraea macarista macarista* (Sharpe)	FDS
Nymphalidae	Heliconiinae	*Acraea natalica natalica* (Boisduval)	N
Nymphalidae	Heliconiinae	*Acraea neobule* (Doubleday)	WSS
Nymphalidae	Heliconiinae	*Acraea penelope vitrea* (Eltringham)	FDS
Nymphalidae	Heliconiinae	*Acraea perenna perenna* (Doubleday)	FEW
Nymphalidae	Heliconiinae	*Acraea pharsalus pharsalus* (Ward)	N
Nymphalidae	Heliconiinae	*Acraea poggei nelson* (Grose-Smith & Kirby)	FDS
Nymphalidae	Heliconiinae	*Acraea pudorella pudorella* (Aurivillius)	N
Nymphalidae	Heliconiinae	*Acraea quirinalis* (Grose-Smith)	FDS
Nymphalidae	Heliconiinae	*Acraea semivitrea* (Aurivillius)	FDS
Nymphalidae	Heliconiinae	*Acraea servona* (Godart)	FDS
Nymphalidae	Heliconiinae	*Acraea sotikensis* (Sharpe)	FDS
Nymphalidae	Heliconiinae	*Acraea uvui uvui* (Grose-Smith)	FEW
Nymphalidae	Heliconiinae	*Acraea ventura ochrascens* (Hewitson)	SWSS
Nymphalidae	Heliconiinae	*Acraea viviana* (Staudinger)	FEW
Nymphalidae	Heliconiinae	*Acraea zetes zetes* (Linnaeus)	WSS
Nymphalidae	Heliconiinae	*Acraea zonata* (Hewitson)	N
Lycaenidae	Polyommatinae	*Actizera lucida lucida* (Trimen)	WSS
Lycaenidae	Polyommatinae	*Actizera stellata* (Trimen)	OHPS
Lycaenidae	Lipteninae	*Alaena picata* (Sharpe)	N
Lycaenidae	Theclinae	*Aloeides conradsi* (Aurivillius)	N
Nymphalidae	Danainae	*Amauris albimaculata* (Butler)	FDS
Nymphalidae	Danainae	*Amauris hecate hecate* (Butler)	FDS
Nymphalidae	Danainae	*Amauris niavius niavius* (Linnaeus)	WSS
Nymphalidae	Danainae	*Amauris tartarea* (Mabille)	FEW
Nymphalidae	Danainae	*Amauris ochlea* (Boisduval)	FEW
Nymphalidae	Charaxinae	*Antanartia delius delius* (Drury)	FDS
Lycaenidae	Polyommatinae	*Anthene amarah amrah* (Guérin-Méneéville)	OHPS
Lycaenidae	Polyommatinae	*Anthene butleri* (Oberthür)	WSS
Lycaenidae	Polyommatinae	*Anthene kampala*	FEW
Lycaenidae	Polyommatinae	*Anthene larydas* (Cramer)	FEW
Lycaenidae	Polyommatinae	*Anthene lunulata* (Trimen)	FEW

Table 2: Continued.

Family	Subfamily	Species	E-cat
Lycaenidae	Polyommatinae	*Anthene schoutedeni* (Huelstaert)	FDS
Pieridae	Pierinae	*Appias sylvia ugandensis* (Bernardi)	FDS
Nymphalidae	Biblidinae	*Ariadne albifascia* (Joicey & Talbot)	FDS
Nymphalidae	Biblidinae	*Ariadne enotrea suffusa* (Joicey & Talbot)	FEW
Nymphalidae	Biblidinae	*Ariadne pangenstecheri* (Suffert)	FEW
Hesperiidae	Hesperiinae	*Artitropa milleri milleri* (Holland)	WSS
Lycaenidae	Theclinae	*Axiocerses harpax ugandana* (Clench)	N
Lycaenidae	Polyommatinae	*Azanus natalensis* (Trimen)	MS
Lycaenidae	Polyommatinae	*Azanus ubaldus* (Cramer)	MS
Pieridae	Pierinae	*Belenois creona* (Cramer)	N
Pieridae	Pierinae	*Belenois raffrayi extendens* (Joicey & Talbot)	FDS
Pieridae	Pierinae	*Belenois solilucus* (Butler)	FEW
Pieridae	Pierinae	*Belenois aurota* (Fabricius)	MS
Pieridae	Pierinae	*Belenois creona severina* (Stol)	MS
Pieridae	Pierinae	*Belenois subeida sylvander* (Grose-Smith)	N
Pieridae	Pierinae	*Belenois theora theora* (Doubleday)	FEW
Pieridae	Pierinae	*Belenois thysa* (Hopffer)	FEW
Nymphalidae	Satyrinae	*Bicyclus buea* (Strand)	FDS
Nymphalidae	Satyrinae	*Bicyclus funebris* (Guérin-Méneville)	FDS
Nymphalidae	Satyrinae	*Bicyclus mollitia*	SWSS
Nymphalidae	Satyrinae	*Bicyclus safitza safitza* (Hewitson)	WSS
Nymphalidae	Satyrinae	*Bicyclus abnormis* (Dudgeon)	WSS
Nymphalidae	Satyrinae	*Bicyclus anynana anynana* (Butler)	OHPS
Nymphalidae	Satyrinae	*Bicyclus campina ocelligera* (Butler)	N
Nymphalidae	Satyrinae	*Bicyclus campinus* (Aurivillius)	SWSS
Nymphalidae	Satyrinae	*Bicyclus campus* (Karsch)	N
Nymphalidae	Satyrinae	*Bicyclus dentatus* (Sharpe)	FEW
Nymphalidae	Satyrinae	*Bicyclus ena* (Hewitson)	OHPS
Nymphalidae	Satyrinae	*Bicyclus evadne elionas* (Hewitson)	N
Nymphalidae	Satyrinae	*Bicyclus golo* (Aurivillius)	FDS
Nymphalidae	Satyrinae	*Bicyclus hewitsoni* (Doumet)	FDS
Nymphalidae	Satyrinae	*Bicyclus istaris* (Plötz)	FEW
Nymphalidae	Satyrinae	*Bicyclus jefferyi* (Fox)	FEW
Nymphalidae	Satyrinae	*Bicyclus kenia* (Rogernhofer)	FDS
Nymphalidae	Satyrinae	*Bicyclus mandanes* (Hewitson)	FDS
Nymphalidae	Satyrinae	*Bicyclus mollitia* (Karsch)	N
Nymphalidae	Satyrinae	*Bicyclus sandace* (Hewitson)	FDS
Nymphalidae	Satyrinae	*Bicyclus saussurei saussurei* (Condamin)	FDS
Nymphalidae	Satyrinae	*Bicyclus smithi smithi* (Aurivillius)	FDS
Nymphalidae	Satyrinae	*Bicyclus sophrosyne sophrosyne* (Plötz)	SWSS
Nymphalidae	Satyrinae	*Bicyclus* sp.1	N
Nymphalidae	Satyrinae	*Bicyclus* sp.2	N
Nymphalidae	Satyrinae	*Bicyclus vulgaris* (Butler)	WSS
Hesperiidae	Hesperiinae	*Borbo fatuellus fatuellus* (Hopffer)	WSS
Hesperiidae	Hesperiinae	*Borbo holtzii* (Plötz)	OHPS
Hesperiidae	Hesperiinae	*Borbo kaka* (Evans)	FDS
Nymphalidae	Biblidinae	*Byblia anvatara acheloia* (Wallengren)	MS
Nymphalidae	Biblidinae	*Byblia ilithyia* (Drury)	N
Lycaenidae	Polyommatinae	*Cacyreus palemon palemon* (Stoll)	OHPS
Lycaenidae	Hesperiinae	*Caenides luehderi laura* (Evans)	N
Pieridae	Coliadinae	*Catopsilia florella* (Fabricius)	MS

Table 2: Continued.

Family	Subfamily	Species	E-cat
Hesperiidae	Pyrginae	*Celaenorrhinus galenus* (Fabricius)	FDS
Hesperiidae	Pyrginae	*Celaenorrhinus ovalis* (Evans)	N
Hesperiidae	Pyrginae	*Celaenorrhinus proxima proxima* (Mabille)	FDS
Nymphalidae	Charaxinae	*Charaxes acuminatus* (Thurau)	FDS
Nymphalidae	Charaxinae	*Charaxes anticlea suna* (van Someren)	FEW
Nymphalidae	Charaxinae	*Charaxes aubyni ecketti*	N
Nymphalidae	Charaxinae	*Charaxes bipunctatus ugandensis* (van Someren)	FDS
Nymphalidae	Charaxinae	*Charaxes brutus* (Cramer)	FEW
Nymphalidae	Charaxinae	*Charaxes chepalungu* (van Someren)	N
Nymphalidae	Charaxinae	*Charaxes contrarius* (Weymer)	N
Nymphalidae	Charaxinae	*Charaxes dilutusngonga*	FDS
Nymphalidae	Charaxinae	*Charaxes etesipe* (Rothschild)	FEW
Nymphalidae	Charaxinae	*Charaxes ethalion nyanzae* (de Boisduval)	WSS
Nymphalidae	Charaxinae	*Charaxes etheocles* (van Someren & Jackson)	FDS
Nymphalidae	Charaxinae	*Charaxes etheocles etheocles* (Cramer)	FDS
Nymphalidae	Charaxinae	*Charaxes eupale latimargo* (Joicey & Talbot)	N
Nymphalidae	Charaxinae	*Charaxes guderiana rabaiensis* (Poulton)	N
Nymphalidae	Charaxinae	*Charaxes hildebrandti hildebrandti* (Dewitz)	FDS
Nymphalidae	Charaxinae	*Charaxes lactetinctus lactetinctus* (Karsch)	OHPS
Nymphalidae	Charaxinae	*Charaxes lucretius maximus* (van Someren)	FDS
Nymphalidae	Charaxinae	*Charaxes nobilis claudei* (le Moult)	FDS
Nymphalidae	Charaxinae	*Charaxes numenes numenes* (Hewitson)	FEW
Nymphalidae	Charaxinae	*Charaxes plantroui* (Minig)	N
Nymphalidae	Charaxinae	*Charaxes pleione* (Godart)	N
Nymphalidae	Charaxinae	*Charaxes protoclea azota* (Hewitson)	FEW
Nymphalidae	Charaxinae	*Charaxes smaragdalis butleri* (Rothschild)	FDS
Nymphalidae	Charaxinae	*Charaxes tiridates tiridates* (Cramer)	FDS
Nymphalidae	Charaxinae	*Charaxes varanes vologeses* (Mabille)	WSS
Nymphalidae	Charaxinae	*Charaxes viola picta* (van Someren & Jackson)	OHPS
Nymphalidae	Charaxinae	*Charaxes xiphares*	FDS
Nymphalidae	Charaxinae	*Charaxes zoolina zoolina* (Westwood)	OHPS
Lycaenidae	Polyommatinae	*Chilades kedonga* (Grose-Smith)	OHPS
Hesperiidae	Hesperiinae	*Chondrolepsis niveicornis niveicornis* (Plötz)	FDS
Lycaenidae	Lipteninae	*Cocyreus lingens*	N
Hesperiidae	Coeliadinae	*Coeliades anchises anchises* (Gerstäcker)	MS
Hesperiidae	Coeliadinae	*Coeliades chalybe* (Westwood)	FDS
Hesperiidae	Coeliadinae	*Coeliades forestan forestan* (Stoll)	WSS
Hesperiidae	Coeliadinae	*Coeliades hanno*	FDS
Nymphalidae	Satyrinae	*Coenyropsis carcassoni* (Kielland)	OHPS
Pieridae	Coliadinae	*Colias electo pseudohecate* (Berger)	MS
Pieridae	Pierinae	*Colotis antevippe zera* (Lucas)	OHPS
Pieridae	Pierinae	*Colotis aurigineus* (Butler)	WSS
Pieridae	Pierinae	*Colotis chrysonome* (Klug)	OHPS
Pieridae	Pierinae	*Colotis danae* (Fabricius)	WSS
Pieridae	Pierinae	*Colotis euippe* (Linnaeus)	WSS
Pieridae	Pierinae	*Colotis venosus* (Staudinger)	OHPS
Lycaenidae	Polyommatinae	*Cupidopsis cissus* (Godart)	WSS
Lycaenidae	Polyommatinae	*Cupidopsis jobates jobates* (Hopffer)	WSS
Nymphalidae	Limenitidinae	*Cymothoe elgesta confusa* (Aurivillius)	N
Nymphalidae	Nymphalinae	*Cymothoe lurida butleri* (Grünberg)	FDS
Nymphalidae	Nymphalinae	*Cymothoe althea althea*	N

Table 2: Continued.

Family	Subfamily	Species	E-cat
Nymphalidae	Limenitidinae	*Cymothoe herminia johnstoni* (Butler)	FDS
Nymphalidae	Limenitidinae	*Cymothoe hobarti hobarti* (Butler)	FDS
Nymphalidae	Limenitidinae	*Cymothoe weymeri weymeri* (Suffert)	N
Nymphalidae	Danainae	*Danaus chrysippus chrysippus* (Linnaeus)	MS
Nymphalidae	Danainae	*Danaus dorripus* (Klug)	MS
Pieridae	Pierinae	*Dixeia orbona vidua* (Butler)	WSS
Pieridae	Pierinae	*Dixeia pigea* (Boisduval)	WSS
Pieridae	Liphyrinae	*Dixeia spilleri spilleri* (Spiller)	N
Lycaenidae	Polyommatinae	*Eicochrysops messapus* (Bethune&Baker)	N
Lycaenidae	Polyommatinae	*Eicochrysops Hippocrates* (Fabricius)	N
Lycaenidae	Polyommatinae	*Eicochrysops masai* (Bethune-Baker)	OHPS
Hesperiidae	Pyrginae	*Eretis lugens* (Rogenhofer)	WSS
Pieridae	Coliadinae	*Eronia cleodora* (Hubne)	OHPS
Lycaenidae	Polyommatinae	*Euchrysops nilotica* (Aurivillius)	N
Lycaenidae	Polyommatinae	*Euchrysops malathana* (Boisduval)	OHPS
Lycaenidae	Polyommatinae	*Euchrysops osiris* (Hopffer)	WSS
Lycaenidae	Polyommatinae	*Euchrysops subpallida* (Bethune-Baker)	N
Nymphalidae	Nymphalinae	*Euphaedra preussi preussi* (Staudinger)	N
Pieridae	Coliadinae	*Eurema brigitta brigitta* (Stoll)	MS
Pieridae	Coliadinae	*Eurema desjardinsi oberthuri* (Mabille)	MS
Pieridae	Coliadinae	*Eurema floricola orientis* (Butler)	FDS
Pieridae	Coliadinae	*Eurema hecabe solifera* (Butler)	MS
Pieridae	Coliadinae	*Eurema regularis regularis* (Butler)	WSS
Pieridae	Coliadinae	*Eurema senegalensis* (Boisduval)	FDS
Nymphalidae	Nymphalinae	*Eurytela dryope angulate* (Aurivillius)	WSS
Nymphalidae	Nymphalinae	*Eurytela alinda* (Mabille)	N
Nymphalidae	Nymphalinae	*Eurytela hiarbas lita* (Rothschild & Jordan)	FEW
Hesperiidae	Hesperiinae	*Freyeria trochylus* (Freyer)	WSS
Hesperiidae	Hesperiinae	*Gegenes hottentota* (Latreille)	OHPS
Hesperiidae	Hesperiinae	*Gegenes niso brevicornis* (Plötz)	N
Hesperiidae	Hesperiinae	*Gegenes pumilio gambica* (Mabille)	OHPS
Nymphalidae	Satyrinae	*Gnophodes betsimena diversa* (Trimen)	FDS
Papilionidae	Papilioninae	*Graphium policenes* (Cramer)	N
Papilionidae	Papilioninae	*Graphium antheus*	FEW
Papilionidae	Papilioninae	*Graphium leonidas Leonidas* (Fabricius)	MS
Papilionidae	Papilioninae	*Graphium polistratus* (Grose-smith)	FEW
Nymphalidae	Satyrinae	*Hallelesis asochis*	N
Nymphalidae	Satyrinae	*Hallelesis halyma*	N
Nymphalidae	Satyrinae	*Halocerina angulata*	N
Nymphalidae	Nymphalinae	*Hamanumida daedalus* (Fabricius)	WSS
Nymphalidae	Satyrinae	*Henotesia peitho* (Plötz)	WSS
Nymphalidae	Satyrinae	*Henotesia perpicua* (Trimen)	OHPS
Nymphalidae	Nymphalinae	*Hypokooelates ugandae* (Talbot)	N
Nymphalidae	Nymphalinae	*Hypolimnas misippus* (Linnaeus)	MS
Nymphalidae	Nymphalinae	*Hypolimnas salmacis magnifica* (Rothschild)	FDS
Lycaenidae	Theclinae	*Hypolycaena hatita ugandae* (Sharpe)	FDS
Lycaenidae	Theclinae	*Iolaus poultoni* (Riley)	FDS
Nymphalidae	Heliconiinae	*Issoria hanningtoni jeanneli* (Bernardi)	FEW
Nymphalidae	Nymphalinae	*Junonia natalica* (Felder)	FEW
Nymphalidae	Nymphalinae	*Junonia chorimene* (Guérin-Ménéville)	OHPS
Nymphalidae	Nymphalinae	*Junonia eonone eonone* (Linnaeus)	WSS

Table 2: Continued.

Family	Subfamily	Species	E-cat
Nymphalidae	Nymphalinae	*Junonia hierta cebrene* (Trimen)	MS
Nymphalidae	Nymphalinae	*Junonia orithya madagascariensis* (Guenee)	N
Nymphalidae	Nymphalinae	*Junonia sophia infracta* (Butler)	N
Nymphalidae	Nymphalinae	*Junonia stygia gregorii* (Butler)	FEW
Nymphalidae	Nymphalinae	*Junonia terea elgiva* (Drury)	WSS
Nymphalidae	Nymphalinae	*Junonia westermannii suffusa* (Rothschild & Jordan)	FDS
Hesperiidae	Hesperinae	*Kedestes rogersi* (Druce)	OHPS
Lycaenidae	Polyommatinae	*Lepidochrysops desmondi* (Stempffer)	N
Lycaenidae	Polyommatinae	*Lepidochrysops elgonae* (Stempffer)	N
Lycaenidae	Polyommatinae	*Lepidochrysops cf jansei* (van Someren)	WSS
Lycaenidae	Polyommatinae	*Lepidochrysops kitale* (Stempffer)	N
Lycaenidae	Polyommatinae	*Lepidochrysops parsimon parsimon* (Fabricius)	OHPS
Pieridae	Pierinae	*Leptosia alcesta inalcesta* (Bernardi)	WSS
Pieridae	Pierinae	*Leptosia nupta nupta* (Butler)	FDS
Pieridae	Pierinae	*Leptosia wigginsi* (Dixey)	FDS
Nymphalidae	Libytheinae	*Libythea labdaca* (Westwood)	MS
Lycaenidae	Lipteninae	*Liptena xanthostola xantha* (Grose-Smith)	N
Nymphalidae	Nymphalinae	*Mallika jacksoni* (Sharpe)	OHPS
Nymphalidae	Satyrinae	*Melanitis leda* (Linnaeus)	WSS
Lycaenidae	Lipteninae	*Mimacraeakrausei*	N
Lycaenidae	Lipteninae	*Mimacraea marshalli* (Trimen)	FEW
Hesperiidae	Hesperiinae	*Monza alberti* (Holland)	FDS
Hesperiidae	Hesperiinae	*Monza cretacea* (Snellen)	WSS
Pieridae	Pierinae	*Mylothris aburi* (Collins & Larsen)	N
Pieridae	Pierinae	*Mylothris agathina* (Cramer)	WSS
Pieridae	Pierinae	*Mylothris chloris clarissa* (Butler)	WSS
Pieridae	Pierinae	*Mylothris continua continua* (Aurivillius)	FDS
Pieridae	Pierinae	*Mylothris hilara* (Karsch)	FDS
Pieridae	Pierinae	*Mylothris ochracea* (Aurivillius)	N
Pieridae	Pierinae	*Mylothris sjoestedti sjoestedti* (Aurivillius)	FDS
Lycaenidae	Theclinae	*Myrina dermaptera nyasae* (Talbot)	OHPS
Pieridae	Pierinae	*Nepheronia argia* (Fabricius)	FDS
Pieridae	Pierinae	*Nepheronia pharis silvanus* (Stoneham)	FDS
Pieridae	Pierinae	*Nepheronia thalassina* (de Boisduval)	FEW
Nymphalidae	Nymphalinae	*Neptidopsis fulgurate* (Rothschild & Jordan)	N
Nymphalidae	Nymphalinae	*Neptidopsis ophione nucleate* (Mabille)	FEW
Nymphalidae	Limenitidinae	*Neptis alta* (Overlaet)	FDS
Nymphalidae	Limenitidinae	*Neptis carcassoni* (van Son)	N
Nymphalidae	Limenitidinae	*Neptis clarei* (Aurivillius)	FDS
Nymphalidae	Limenitidinae	*Neptis jordani* (Neave)	N
Nymphalidae	Limenitidinae	*Neptis katama* (Collins&Larsen)	N
Nymphalidae	Limenitidinae	*Neptis kiriakoffi* (Overlaet)	WSS
Nymphalidae	Limenitidinae	*Neptis laeta* (Overlaet)	WSS
Nymphalidae	Limenitidinae	*Neptis melicerta melicerta* (Hewitson)	FDS
Nymphalidae	Limenitidinae	*Neptis metella metella* (Doubleday)	FEW
Nymphalidae	Limenitidinae	*Neptis morosa* (Overlaet)	WSS
Nymphalidae	Limenitidinae	*Neptis nemetes nemetes* (Hewitson)	FEW
Nymphalidae	Limenitidinae	*Neptis occidentalis occidentalis* (Rothschild)	FDS
Nymphalidae	Limenitidinae	*Neptis ochracea milbraedi* (Gaede)	FDS
Nymphalidae	Limenitidinae	*Neptis penningtoni*	N
Nymphalidae	Limenitidinae	*Neptis poultoni* (Eltringham)	FDS

Table 2: Continued.

Family	Subfamily	Species	E-cat
Nymphalidae	Limenitidinae	*Neptis saclava marpessa* (Hopffer)	WSS
Nymphalidae	Limenitidinae	*Neptis strigata* (Aurivillius)	FDS
Nymphalidae	Limenitidinae	*Neptis woodwardi woodwardi* (Sharpe)	FDS
Lycaenidae	Polyommatinae	*Oboronia punctatus*	N
Lycaenidae	Lipteninae	*Ornipholidotos peucetia peuceda* (Grose-Smith)	FDS
Papilionidae	Papilioninae	*Papilio bromius* (Suffert)	FEW
Papilionidae	Papilioninae	*Papilio dardanus polytropus* (Brown)	WSS
Papilionidae	Papilioninae	*Papilio demodocus* (Esper)	MS
Papilionidae	Papilioninae	*Papilio lormieri*	FDS
Papilionidae	Papilioninae	*Papilio nireus lyaeus* (Doubleday)	FEW
Papilionidae	Papilioninae	*Papilio nobilis* (Rogenhofer)	FDS
Papilionidae	Papilioninae	*Papilio ophidicephalus* (Oberthür)	N
Papilionidae	Papilioninae	*Papilio phorcas* (Cramer)	FDS
Papilionidae	Papilioninae	*Papilio zoroastres joiceyi* (Gabriel)	FDS
Papilionidae	Hesperinae	*Paracleros biguttulus* (Mabille)	FEW
Hesperiidae	Hesperinae	*Pardaleodes incerta* (Snellen)	FDS
Hesperiidae	Hesperinae	*Pardaleodes sator pusiella* (Mabille)	FDS
Nymphalidae	Heliconiinae	*Pardopsis punctatissima* (Boisduval)	WSS
Lycaenidae	Lipteniinae	*Pentila tachyroides tachyroides* (Dewitz)	N
Lycaenidae	Lipteniinae	*Pentila pauli clarensis* (Neave)	N
Nymphalidae	Heliconiinae	*Phalanta phalantha* (Rothschild & Jordan)	MS
Pieridae	Pierinae	*Pinacopteryx eriphia melanarge* (Butler)	OHPS
Pieridae	Pierinae	*Pontia helice johnstoni* (Crowley)	MS
Nymphalidae	Nymphalinae	*Precis antilope* (Feishamel)	OHPS
Nymphalidae	Nymphalinae	*Precis archesia* (Cramer)	OHPS
Nymphalidae	Nymphalinae	*Precis ceryne ceryne* (Boiduval)	SWSS
Nymphalidae	Nymphalinae	*Precis coelestina*	FEW
Nymphalidae	Nymphalinae	*Precis octavia sesamus* (Trimen)	WSS
Nymphalidae	Nymphalinae	*Precis tugela* (Trimen)	FEW
Hesperiidae	Hesperinae	*Prosopalpus debilis* (Evans)	N
Hesperiidae	Hesperinae	*Protogoniomorpha temora*	N
Nymphalidae	Nymphalinae	*Pseudacraea eurytus eurytus* (Linnaeus)	FDS
Nymphalidae	Nymphalinae	*Pseudacraea lucretia protracta* (Cramer)	FEW
Nymphalidae	Nymphalinae	*Pseudacraea boisduvali* (Doubleday)	N
Nymphalidae	Nymphalinae	*Pseudargynnis hegemone* (Godart)	FEW
Nymphalidae	Nymphalinae	*Pseudacraea lucretia expansa* (Butler)	N
Nymphalidae	Nymphalinae	*Salamis cacta cacta fbricius*	FDS
Nymphalidae	Nymphalinae	*Salamis parhassus* (Drury)	FEW
Nymphalidae	Nymphalinae	*Sallya boisduvali* (Wallengren)	MS
Nymphalidae	Nymphalinae	*Sallya garega garega* (Karsch)	MS
Nymphalidae	Nymphalinae	*Sallya natalensis* (Boisduval)	MS
Nymphalidae	Nymphalinae	*Sallya occidentalum occidentalium* (Mabille)	MS
Nymphalidae	Nymphalinae	*Sallya umbrina* (Karsch)	MS
Hesperiidae		*Sarangesa laelius* (Mabille)	WSS
Lycaenidae	Pyrginae	*Liptena maculata* (Mabille)	OHPS
Hesperiidae	Pyrginae	*Septena xanthostola xantha*	N
Hesperiidae	Pyrginae	*Spialia dromus* (Plötz)	N
Hesperiidae	Pyrginae	*Spialia mafa higginsi* (Evans)	N
Hesperiidae	Pyrginae	*Spialia mangana* (Rebel)	N
Hesperiidae	Pyrginae	*Spialia ploetzi ploetzi* (Aurivillius)	N
Hesperiidae	Pyrginae	*Spialia spio* (Linnaeus)	OHPS

Table 2: Continued.

Family	Subfamily	Species	E-cat
Hesperiidae	Hesperiinae	*Tagiades flesus*	N
Lycaenidae	Lipteninae	*Tetrarhanis diversa ilala* (Riley)	N
Nymphalidae	Danainae	*Tirumala formosa morgeni* (Honrath)	FEW
Nymphalidae	Danainae	*Tirumala petiverana* (Doubleday & Hewitson)	MS
Lycaenidae	Polyommatinae	*Triclema nigeriae* (Aurivillius)	FEW
Lycaenidae	Polyommatinae	*Tuxentius margaritaceus margaritaceus* (Sharpe)	WSS
Lycaenidae	Polyommatinae	*Uranothauma falkensteini* (Dewitz)	N
Nymphalidae	Nymphalinae	*Vanessa cardui cardui* (Linnaeus)	N
Nymphalidae	Nymphalinae	*Vanesula milca latifasciata* (Joicey & Talbot)	FEW
Nymphalidae	Satyrinae	*Ypthima albida albida* (Butler)	FEW
Nymphalidae	Satyrinae	*Ypthima antennata antennata* (van Son)	OHPS
Nymphalidae	Satyrinae	*Ypthima asterope asterope* (Klug)	OHPS
Nymphalidae	Satyrinae	*Ypthima granulosa* (Butler)	OHPS
Nymphalidae	Satyrinae	*Ypthima rhodesiana* (Carcasson)	N
Hesperiidae	Hesperiinae	*Zezonia zeno* (Trimen)	FEW
Lycaenidae	Polyommatinae	*Zizeeria knysna* (Trimen)	WSS
Lycaenidae	Polyommatinae	*Zizina antanossa* (Mabille)	WSS
Lycaenidae	Polyommatinae	*Zizula hylax* (Fabricius)	WSS

Codes for different ecological habitat categories. E-cat: Ecological habitat category. FEW (f/m): "forest edge/woodland species"; FDS (F): "forest-dependent species"; MS (m): "migratory species"; OHPS (O): "open habitat specialist species"; SWSS (S): "swamp/wetland specialist species"; WSS (W): "widespread species" that are frequent fallows/grassland habitat users; N (U): "uncertain ecological category"—species of unknown (un-described) habitat preference.

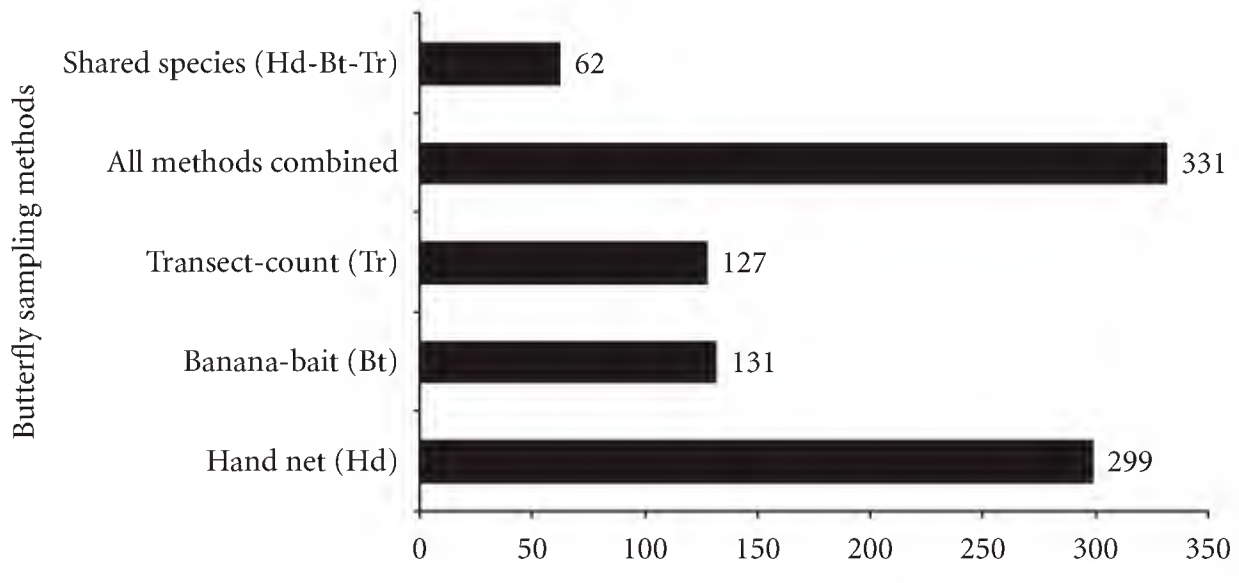

Figure 3: Number of butterfly species obtained using different sampling methods in farmlands of central Uganda during 2006.

small-scale grazing fields, hedgerows, and annual/biannual crop fields in the surrounding of forest reserves. Axis 2 was strongly significantly and positively related to environmental factors such as hedgerows, annual/biannual crop fields in the surrounding of forest reserves. Axis 1 was also negatively influenced by environmental factors such as large scale of tea plantations in vicinity of wetlands/swamps, but positively related to fallows. The fourth axis did not have high loadings but was observed to be frequently correlated (positively) to some other environmental factors: fallows and sugar plantation (Table 6). Environmental factors that were identified in the CCA as determinants for butterflies (Table 5) were also among the environmental factors observed to be significantly ($P < 0.05$, LSD test) associated with high species richness and abundance of butterflies (Table 6) in the coffee-banana agroforest systems. Across studies sites and sampling rounds, these habitats attracted a high number of butterflies both during the rainy and the dry seasons. Practically, few butterfly species were closely associated with "determinant environmental factors" previously identified in the CCA (Table 4) including swampy habitats, fallows, forest plantations, woodlands, stream-edges, tea plantations, and sugar plantations. For example, *Belenois subeida, Bicyclus safitza, Melanitis leda, Charaxes varanes,* and so forth appeared to be closely associated with fallows (Figure 4). Some of the species closely associated with swamp habitats and forest plantations may be considered as "farmland habitat specialists." The rest of species may be "generalist farmland habitats users."

Table 3: Butterfly species richness, abundance (individuals/2 ha/site), and diversity (H′) in 26 different study sites in the coffee-banana farming system in central Uganda in 2006.

Clusters	Sites	Land use intensity gradients	Species richness	Abundance	Diversity
Bujjagali	Bukose	High	18.17 ± 1.56c	287.17 ± 8.38b	2.31 ± 0.28b
	Namizi-east	High	23.41 ± 3.06b	510.13 ± 11.33b	2.24 ± 0.17e
	Namizi-west	High	14.61 ± 0.69c	230.23 ± 8.11b	2.67 ± 0.21d
	Nawangoma	High	17.46 ± 0.71c	274.33 ± 6.09b	2.12 ± 0.11f
Kalagi	Bamusuta	Medium	20.54 ± 0.63c	392.36 ± 8.72b	2.95 ± 0.21c
	Kifu	Medium	29.13 ± 3.39b	541.57 ± 12.03b	3.35 ± 0.33b
	Kimwanyi	Medium	31.81 ± 3.59b	562.88 ± 12.51b	3.36 ± 0.37b
	Kiweebwa	Medium	30.23 ± 1.51b	1062.6 ± 15.93a	3.24 ± 0.25b
Kamuli	Naikesa	High	12.45 ±03 2d	151.87 ± 3.37c	1.81 ± 0.22g
	Namulekya	High	16.62 ± 0.83c	213.34 ± 7.74b	1.87 ± 0.20g
Kaweri	Luwunga	Very high	18.37 ± 0.51c	150.52 ± 3.34c	1.69 ± 0.21g
	Nonve	Very high	11.76 ± 0.58d	141.49 ± 3.14c	1.61 ± 0.19g
Lugazi	Kasaku	Very high	26.22 ± 2.31b	780.68 ± 16.67a	3.22 ± 0.12b
	Sugar	Very high	15.89 ± 0.79c	269.57 ± 8.99b	2.13 ± 0.32f
Mabira	Bulyasi	Medium	22.16 ± 1.11c	409.58 ± 9.10b	3.09 ± 0.21c
	Kinoni	Medium	16.44 ± 1.82c	316.12 ± 7.02b	2.64 ± 0.31d
Masaka	Kasaala	Medium	19.19 ± 2.12c	421.48 ± 9.37b	2.44 ± 0.16e
	Katwadde	Medium	21.22 ± 2.36c	426.59 ± 9.48b	2.63 ± 0.24a
	Kiwaala	Medium	28.87 ± 3.23b	625.57 ± 19.90a	3.35 ± 0.27b
	Mpugwe	Medium	19.24 ± 2.13c	217.69 ± 7.840b	2.51 ± 0.33d
Mpigi	Lukalu	Medium	44.65 ± 4.95a	716.67 ± 17.86a	3.41 ± 0.25a
	Mpanga	Medium	38.19 ± 4.23a	678.57 ± 17.13a	3.56 ± 0.26a
Nakaseke	Kimuli	Low	30.64 ± 3.41b	918.27 ± 21.71a	3.71 ± 0.27a
	Kyetume	Low	20.87 ± 2.31c	368.67 ± 8.87b	3.14 ± 0.29c
	Lukumbi	Low	13.67 ± 2.51c	282.88 ± 7.44b	3.12 ± 0.23c
	Segalye	Low	27.65 ± 3.06b	482.98 ± 11.67b	3.01 ± 0.23c

Within columns, means followed by the same letter are not significantly different at 5% probability level (LSD test) test.

3.3. Effects of Farm Management Regimes on Butterflies. Although large monocultures (tea, sugar, coffee plantations) that are intensively managed appeared among important factors in the CCA analysis (Table 4), the species richness, abundance and diversity of butterflies were significantly ($P < 0.05$, LSD test) higher in traditional small-scale polyculture fields than in arge monoculture plantations (Table 7). These results indicated that small scale polyculture fields supported richer butterfly communities than did large monoculture (modernized fields).

4. Discussion

4.1. Determinants of Butterfly Species Distribution in Coffee-Banana Farming Systems. In this study, it is predicted that there would be no statistical differences among sites with different land uses, seminatural habitats, and farm management systems for the abundance, richness and diversity of butterflies since farmland habitats are subjected to constant anthropogenic disturbances. The results did not confirm the hypothesis since there were significant differences in species richness and abundance among the 26 study sites with different land-use types, habitat types and farm management regimes. Sites that hosted higher species richness and diversity were study sites that were riparian forest reserves or sites for which agricultural matrices were surrounded by forest patches and wetlands (see agroforests characteristics in Table 1). In contrast, the least diverse study sites or sites characterized by low butterfly species richness were also study sites of low habitat heterogeneity. These were also sites dominated by high management intensities (sites subjected to high cropping intensity) or sites located in regions with high land-use intensity gradients.

Overall butterfly species richness and density varied seasonally, with more species and higher densities in the rainy seasons (wet months) compared with the dry season (dry months). It is likely that seasonal changes in species

Table 4: Summary of canonical correspondence analysis (CCA) ordination results relating butterfly community to environmental variables measured across 26 study sites in 2006.

Summary					
Axes	1	2	3	4	Total inertia
Eigenvalues	0.267	0.243	0.239	0.208	3.549
Species-environment correlations	0.955	0.950	0.883	0.942	
Cumulative percentage variance					
of species data	7.51	14.34	20.92	26.68	
of species-environment relation	16.32	31.19	45.38	57.72	
Sum of all eigenvalues					3.549
Sum of all canonical eigenvalues					1.639
Summary of Monte Carlo test					
Test of significance of first canonical axis					Eigenvalue = 0.269 F-ratio = 1.218 P value = 0.9469
Test of significance of all canonical axes					Trace = 1.638 F-ratio = 1.289 P value = 0.0159

Table 5: Coefficient of correlations between the matrix of environmental (habitat/land-use) variables and the 4 axes of the CCA ordination scores. Different levels of significance of Spearman rank coefficients of correlation of the environmental variables with axes: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns: $P > 0.05$. Correlations indicate the degree to which species occurrence and distributions correspond to changes in each variable in the environmental matrix.

Variable codes	Definition of environmental (habitat/land use) variables that were measured concurrently with butterfly surveys	AX1	AX2	AX3	AX4
Ag fi	Small-scale agroforestry fields with fruit tree species	−0.025 ns	−0.2264 ns	0.6265***	−0.2697 ns
Pla cof	Large coffee plantations in the margins of native forest fragments	0.158 ns	0.0732 ns	0.5488**	0.0641 ns
Fo p	Annual/biannual crop fields in the surrounding of forest reserves	0.1809 ns	0.5947**	0.4686*	0.2083 ns
Gr pl	Small-scale grazing fields (cows, goats, sheep) + hedgerows	0.2799 ns	0.5748**	0.4771*	0.1021 ns
Fall	Fallows (young, old, forest and mixed fallows)	0.521**	−0.1895 ns	−0.0199 ns	0.4696*
SS field	Small-scale monoculture/polyculture fields of annual/biannual crops	0.3531 ns	0.2615 ns	0.631**	−0.0049 ns
Sugar	Large sugar plantations with large water canals	−0.1413 ns	−0.0512*	−0.2619 ns	0.4583*
Sw hbt	Swampy habitats + reclaimed wetlands + stream-edges	0.1242 ns	−0.495*	0.5256**	−0.2101 ns
Tea	Large-scale of tea plantations in vicinity of wetlands/swamps	−0.4515*	−0.001 ns	−0.3991*	0.0468 ns
Wdld	Woodlots (Eucalyptus/Pine plantations) + woodlands + grasslands	0.0325 ns	0.452*	0.5594**	0.2514 ns

richness and density of butterflies may be attributed to monthly variations in climatic factors such as temperature and precipitation. The seasonality in butterfly species occurrence tended not to follow very closely the general patterns of rainfall in central Uganda. However, peak in butterfly species richness appeared to coincide with peaks in flowering of the most butterfly food plants.

In this study, it was observed that sites with higher cover of pasturelands, grasslands, woodlands, fallows, swamps, and stream edges had great diversity, species richness and population density compared to sites with high tree cover in their surroundings. Also, species richness was highest in study sites that were riparian forest fragments and related natural habitats (wetlands). Additionally, it was observed that study sites with higher farm management intensities were less diverse since large monoculture plantations supported less species rich butterfly communities compared to small-scale fields. The significant difference in mean species richness and diversity across sites was also accounted for by the difference in site characteristics (difference in quality of seminatural and natural habitats found within and in the vicinity of fields). The high species richness of butterflies found may be explained by the fact that mosaic of farm landscapes of central Uganda are characterized by diverse habitat types within the heterogeneously used agricultural matrix that contain a variety of host plants and breeding sites required by butterfly species belonging to different ecological groups. Additionally, strongest differences observed in species richness, abundance, and diversity were apparently related to site biophysical characteristics (see agroforests characteristics in Table 1).

The influence of site biophysical characteristics on diversity and abundance of butterflies was also supported by the ordination analysis. The presence of forest reserves,

Table 6: Richness and abundance of butterflies attracted to different habitats (land uses) frequently observed in the coffee-banana farming systems of central Uganda. (Most attractive habitats were those with >20% of weeds/crops/grass/wild plant species blooming or with buds in flowers at the time of visit.)

Foraging habitats: seminatural habitat types/crop-field habitat types	Habitat Frequency	Habitat size range (ha)	*N*	Butterfly species (x + SE)	Butterfly abundance (x + SE)
Field margins associated with or not termite mounds and shrubs/trees/grass species	125	0.05–0.5	159	5.70 ± 0.41d	16.91 ± 3.52c
Boundaries of small-scale polyculture/monoculture fields	96	0.01–0.05	150	3.70 ± 0.81e	27.42 ± 3.65c
Forest reserves in vicinity of small-scale fields and large coffee plantations	25	0.5–40	45	**21.2 ± 2.12a**	**55.83 ± 12.1a**
Woodlands, grasslands	15	0.5–40	30	**16.5 ± 1.07b**	31.44 ± 2.87c
Forest plantations (Pine/Eucalyptus)	11	0.5–30	22	**13.1 ± 0.34b**	24.61 ± 0.21c
Simple and complex traditional agroforests with fruit trees + pulse crops + cereals + root/tuber crops	34	0.05–2.9	119	**14.56 ± 1.21b**	29.34 ± 2.72c
Agroforestry woodlots (with species like *Moringa* sp., *Sesbania* sp., *Leucaena* sp., *Calliandra* sp., etc.)	21	0.05–2	45	3.12 ± 0.55e	19.55 ± 5.61c
Grazing fields, pastures, hedgerows	67	0.05–10	190	9.90 ± 1.87c	17.86 ± 4.67c
Swampy habitats, wetland-edges, streams-edges	54	0.3–5	89	**17.7 ± 2.51b**	**67.87 ± 18.2a**
Abandoned gardens	46	0.02–1	258	10.1 ± 1.12c	41.58 ± 7.67b
Fallows (young, old, and forest fallows)	100	0.03–3	356	**16.4 ± 1.97b**	46.69 ± 8.76b
Herbaceous or unweeded crop fields and unplowed fields	121	0.02–3	367	**16.78 ± 1.56b**	36.89 ± 1.15c
Perennial crops (e.g., large plantations of coffee/sugar cane)	89	0.05–12	600	3.92 ± 0.23e	16.32 ± 1.11c
Perennial crops (banana) grown sole or mixed with coffee and *Vanilla*	65	0.05–15	789	1.18 ± 0.92h	3.268 ± 0.25d
Perennial tree fruit crops (avocado, mango, citrus, lemon, guava, papaya, etc.) grown sole	25	0.03–10	98	3.76 ± 0.98e	16.43 ± 1.13c
Perennial fruit crops (passion fruit) grown sole	12	0.01–3	22	3.12 ± 1.13e	15.65 ± 1.59c
Homegardens of annual vegetables (pumpkin, watermelon, cleome, etc.)	48	0.01–0.5	79	4.62 ± 0.47d	25.78 ± 1.43c
Annual commercial/cash vegetable crops (tomato, egg plant, pepper) grown sole or mixed	38	0.02–6	121	2.11 ± 0.95f	10.21 ± 2.35d
Annual commercial/cash crops (sim-sim, sun flower) grown sole or mixed with legumes	15	0.02–10	48	2.12 ± 0.88f	13.59 ± 2.45d
Annual cereals mixed with pulse crops (beans,cowpea, greengram, soybean): Maize	61	0.04–7	421	2.13 ± 0.81f	5.12 ± 0.59e
Annual cereals mixed with pulse crops: sorghum/millet	19	0.03–6	259	3.17 ± 0.97e	11.67 ± 2.11d
Annual cereals (maize, sorghum, millet) mixed with pulse crops(beans, groundnut): Beans	41	0.02–5	342	2.43 ± 0.31f	9.625 ± 2.61d
Annual cereals mixed with pulse crops: Groundnut, cowpeas	26	0.02–6	403	1.23 ± 0.34h	4.624 ± 0.54e
Bi-annual crops (cassava)	26	0.02–8	198	1.12 ± 0.16h	4.232 ± 0.98e
Annual crops (sweet potato)	51	0.02–3	400	4.32 ± 0.67d	8.963 ± 2.42d
Annual cereals (rice)	16	0.05–15	55	1.13 ± 0.13h	4.651 ± 0.87e
Annual crops (Irish potato)	28	0.05–12	43	2.45 ± 0.41h	12.34 ± 2.76d

Habitat frequency: number of observation cases or number of times the habitat type was encountered across all 26 study sites and all sampling rounds.
Habitat size range (ha): the data show the minimum and the maximum size of the type of habitat encountered during butterfly faunistic surveys.
N: number of samples (butterfly species and individuals) recorded in five sampling rounds across the 26 study sites in 2006.
Within columns, different letters show significant differences of the means at $P = 0.05$ according to LSD test performed after Kruskall Wallis-ANOVA test indicated that the habitat type was significant ($P < 0.001$) for the number of species and individuals attracted.

Table 7: Effect of the farm intensity management system on the richness, abundance, and diversity of butterfly found in the coffee-banana farming system in central Uganda.

Farm management systems	Species richness (number species/site)	Abundance (individuals/site)	Diversity (Shannon-diversity index: H')
Modernized (large-scale fields)	14.21 ± 2.73b	330.2 ± 79.55b	2.28 ± 0.81b
Traditional (small-scale fields)	19.95 ± 4.41a	685.7 ± 231.47a	2.89 ± 1.59a

Within each column, means followed by the same letters are not significantly different at 5%, LSD test.

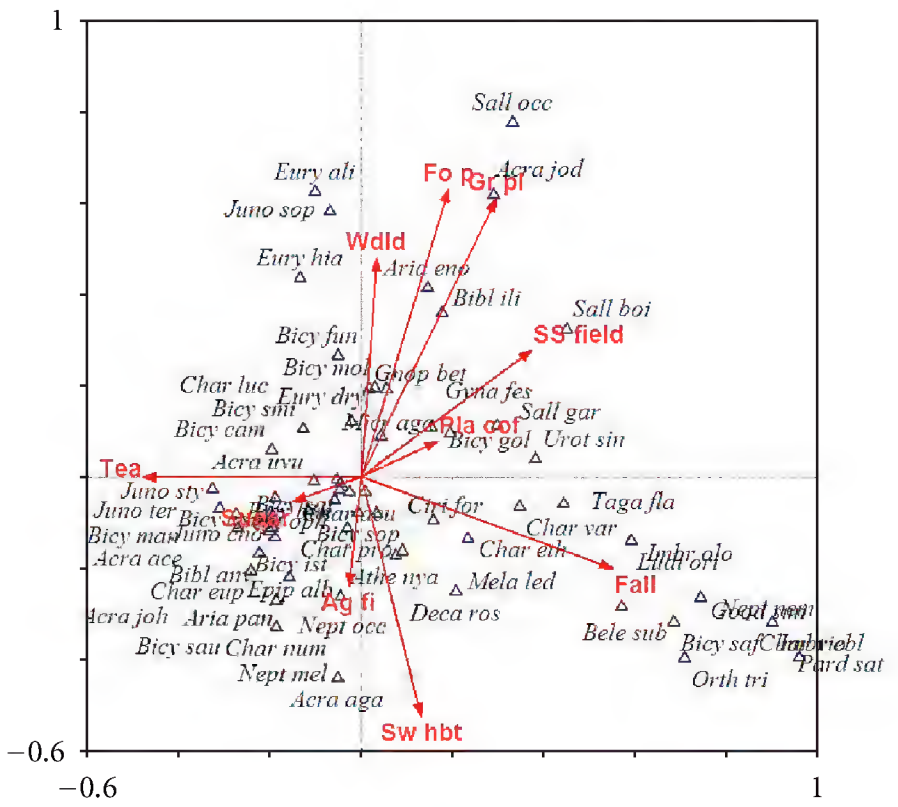

Figure 4: Canonical correspondence analysis (CCA) ordination diagram biplot showing correlation between butterfly specie (*Acra joh, Mela led, and Bicy sau* are short names of specie) and the environmental variables (codes: solid line vector "Tea," "Sw hbt," "Fall," etc.).

woodlands, fallows, hedgerows, woodlots (pine/eucalyptus forest plantations) in the vicinity of fields were environmental factors that appeared to be more important and primary determining the occurrence of foraging butterfly species composition. The ordination analysis demonstrated that the occurrence of different butterfly species in the farmland was strongly positively association with availability of natural and seminatural habitats found in the surrounding of fields. Thus, seminatural and natural habitats are among environmental factors playing key roles in shaping butterfly communities potentially delivering pollination services to wild and cultivated plants in the coffee-banana agroforestry systems of central Uganda.

4.2. Butterfly Assemblages in Tropical Agroforestry Systems. In this study, farmlands were hypothesized not to be suitable habitats for diverse butterfly communities in central Uganda. In contrast to expectations, the results indicated that farmlands of central Uganda supported a rich butterfly fauna and that there were significant ($P < 0.05$) variation in species richness, abundance and diversity among the 26 sites studied. Small-scale polyculture fields and other traditional complex agroforestry systems that were established in the vicinity of forests fragments or traditional simple agroforestry systems with a high cover of seminatural habitats (fallows, swamps, hedgerows) supported more species and individuals of butterflies than other typical cropping systems. These important agroforestry systems supported also a high number of species of butterfly food plants. Several butterfly food plants species were recorded in a previous study [7].

In this study it was observed that farmlands of central Uganda supported species rich-butterfly faunas that are highly seasonal. The high number of butterfly species and individuals recorded in small scale polycultures indicated that a certain number of wild and cultivated plant species were receiving Lepidoptera pollination services of high quality. In other words, several butterfly species occurring in the farmland of central Uganda may be efficient pollinator species on many cultivated and wild plant species (subspecies, land races, genotypes, varieties). In fact, some butterfly species in the Lycaenidae, Nymphalidae family were frequently recorded collecting floral resources on several weeds/crops in bloom that were found in different small scale field where cereals and legumes were mixed. In farmlands of central Uganda, butterflies have been observed being engaged in different activities (perching, flying, mating, foraging, and oviposition). With field experience, some butterfly species were observed making long visits on crop flowers such as cowpeas, beans, groundnut, and egg plants. They made their visits to crop flowers mainly between 9 h00 and 11 h00 and between 14 h00 and 16 h00 (Figure 5).

In central Uganda, *Catopsilia florella, Bicyclus safitza, Junonia Sophia,* and *Acraea acerata* were especially abundant while a much larger number of species were relatively rare (>60% of species recorded), reflecting patterns expected from tropical environments.These species appeared to be well adapted for survival in farmlands of central Uganda. They are well known to be abundant in grassland habitats in Uganda [7]. They are probably typical farmland species as no reports have ever mentioned them among the dominant species of forest ecosystems in Uganda. In addition, *Catopsilia florella* and *Acraea acerata* have been reported by local farmers to be pests, although scientific evidence indicates that only *A. acerata* is a pest of crop species such as sweetpotato [30]. Similar observations were reported by Fernández-Hernández [31] in Cuba.

Farmland butterflies are believed to pollinate many wild and cultivated plant species. Pollination activities of some species are well known. Continuous observations are needed in the future in order to determine the pollination efficiency

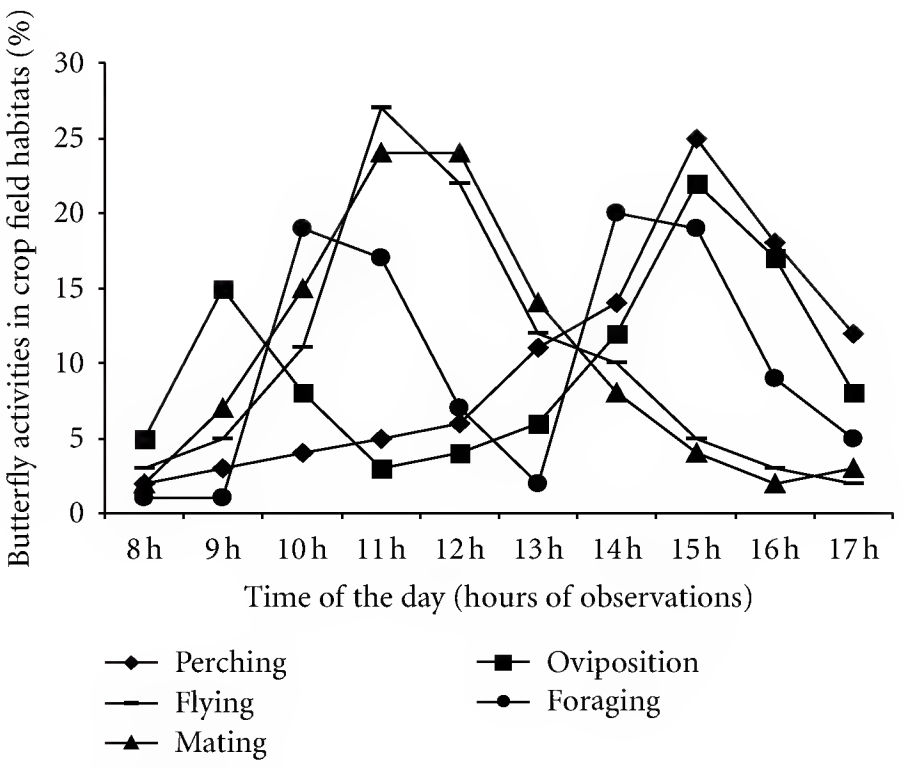

FIGURE 5: Distribution of the daily activities of few butterfly species that were monitored when visiting crop field habitasts in centrtal Uganda in 2006—preliminary results. With author experience, observations were recorded (N = 679 observations conducted on 5 butterfly species).

of all butterfly species on wild and cultivated plant species. This may help in development of strategies for their conservation and management within farmland habitats. The functional status (as pest or as pollinator) of *Catopsilia florella* remains largely unknown. This species disperses locally and in this study, the species was observed foraging frequently on both flowering herbs/weeds and on some crop species.

In central Uganda, farmlands were found to be visited by a high number of species of "forest dependent species" (mainly Nymphalidae).This finding contrasts with observations of Fermon et al. [32] who found that the majority of Nymphalidae species were avoiding farmlands located in the vicinity of forest habitats. Fermon et al. [32] stressed that only a few specialist nymphalid species could visit pine/eucalyptus plantations adjacent to the natural forests in Bossematie zone (Cote D'ivoire). The main reason for central Uganda to support high butterfly diversity dominated by Nymphalidae is the presence of forest patches within agricultural matrices, making central Uganda to appear more like "small degraded forests than just typical agricultural mosaic landscape" [7].

4.3. Patterns of Butterfly Diversity and Abundance in Farmland Habitats. In this study, a great variation in species richness and density among the 26 study sites was observed. The most species-rich site contained more than four times the number of species as the poorest site overall. The highest species richness, population density, and diversity of butterflies were found to be associated with study sites that are riparian forest reserves (see Table 1).

Much as factors governing butterfly community structure and composition in farmlands are not fully known, this result indicates that forest vegetation play a significant role in the distribution, diversity, and density of butterfly species in farmlands of central Uganda. A possible explanation is that butterflies visit farmlands for supplemental nectar resources not found in the adjacent forest ecosystems.

In East Africa, farmlands are expected to be populated by a small number of butterfly species, generally 5 to 50 represented by a relatively high number of individuals. While in this study it was observed that a high number of forest-dependent species (40% of 331 species recorded), including fruit-feeding species, that visited crop fields located in the adjacent; a recent study conducted in Ghana found that very few (<10% of 90 species recorded) fruit-feeding species occurred in the farm bush savannah [33].

Several studies indicate that farmland habitats support poor communities of butterflies [14, 34–36]. However, few studies have highlighted similar findings to those obtained in central Uganda. For example, in Vietnam, Lien and Yuan [37] recorded a higher diversity of butterflies in agricultural habitats [28, 37]. Similar observations were recorded by Lien and Yuan [37] in Costa Rica where high species richness of butterfly was recorded in coffee plantations as compared to adjacent forest remnant fields. In addition, Kitahara [21] recorded higher species richness and diversity in agricultural landscapes in Japan compared to forest fragments. These previous studies show that elsewhere (out of sub-Sahara Africa) farmland habitats can be important for butterflies comparable to forest ecosystems as it was observed in central found in Uganda.

In conclusion, the overall objective of this study was to provide information on butterfly communities to conservation planners and policy makers in central Uganda. The results show that the species diversity of butterfly in the coffee-banana agroforestry systems of central Uganda is much higher than expected. Butterflies visited a variety of habitats and land uses, some of them were better than others to harbor butterflies. Important habitats for farmland butterflies included woodlands, fallows, hedgerows, swampy habitats, abandoned gardens, and homegardens.

Findings from this study indicated that agricultural field sites that were riparian forest fragments/wetlands had the highest species richness and population density of butterflies compared to field sites established far from natural habitats (forests, wetlands). They might support the concept of corridors across large agricultural areas.

Higher forest cover within landscapes had a major positive influence on butterfly biodiversity in agricultural landscapes and related human-modified landscapes in central Uganda.

Although data was collected during five rounds (knowing that five samples may not be enough for robust statistical analysis) conducted in only one year (2006), this study supplied valuable information about the diversity of butterflies in coffee-banana agroforestry areas in central Uganda. The research findings indicated that local coffee-banana agroforestry systems are valuable for butterfly conservation.

A high number of species and individuals were recorded in small scale polycultures. This indicated that some wild and cultivated plant species were potentially receiving pollination

services of high quality from different butterfly species. There is a need to develop appropriate strategies to conserve coffee-banana agroforests to maintain butterfly communities delivering pollination services to crops and wild plants within and nearby crops habitats. The conservation of remnant forest patches through restoration of different habitats, protection from degradation and improvement of the connectivity to larger forest patches and related man-made forest plantations (woodlots, forest plantations) needs to be prioritized by policy makers and land-use planners. Their protection from degradation should be a priority for decision makers since these forest support a rich community of butterflies potentially delivering pollination services to cultivated plants on which human beings depend on for their livelihoods.

Farmers are advised to adopt landscape management and farming practices that are friendly to butterfly species pollinating some of their crops such as practices that can increase high on-farm cover (%) of different types of land uses (polycultures of cereals mixed to legume crops, traditional simple and complex agroforestry systems), of seminatural features (hedgerows, fallows, grasslands, pasturelands, swampy habitats, stream-edges) and related habitats (forest patches, forest fallows, woodlots of eucalyptus, pines) or farming practices that protect and increase the area covered by noncrop habitats within farmed landscape to serve as "butterfly reservoirs." Such uncultivated areas can also support larval host plants and various nectarine plant species that may attract different adult butterfly species in the farmlands.

Farmers are recommended to invest in the protection of habitats to offer habitat and nectar sources to butterflies in the farmland. The protection of natural and seminatural habitats in the coffee-banana farming system may be of different uses: serving as source of butterflies for a butterfly farm. The coffee-banana farming system may also be used as tourist place to see common farmland butterflies species. More practically, they need protection since preliminary observations indicated that some cultivated crops were receiving pollination services [7] from some butterfly species.

Farm management regime had an impact of butterfly diversity such as small scale farms supported highly diverse butterfly communities than did modernize large plantations. Therefore, farmers are encouraged to adopt polyculture cropping systems integrated to good management of floral resources in the margins of fields.

To provide contributions to the conservation of butterfly fauna in agricultural landscapes in Uganda, it is important to determine the pollination efficiency of different butterfly species. The recommended correct sampling method to bring appropriate result is transect count. This method may be a suitable method to assess the diversity of butterfly communities delivering pollination services to wild and cultivated crops in different farmland habitats of central Uganda. The method is recommended to be used for monitoring butterflies, as well as assessing impact of habitat disturbance on community diversity of butterflies. Banana-bait trap method may be recommended for assessing/monitoring fruit feeding community in the area while hand net may be used when aiming at detecting rapidly a high number of species in the habitat.

Acknowledgments

The author is very grateful to Darwin Initiative for funding this study. Also, he is very grateful to farmers for offering their farms for conducting butterfly sampling. The valuable inputs in this work from Professor Philip Nyeko (Makerere University Uganda) and Professor Simon Potts (University of Reading UK) (scientific supervisors) are acknowledged. The author owes his deepest gratitude to Dr. Phil Atkinson (British Trust Ornithology, UK), Dr. Juliet Vickery (RSPB-Cambridge-UG), and Professor Derek Pomeroy (project leaders and scientific advisors) for their financial support. Mr. Maurice Mutabazi is thanked for his assistance in the field. The author acknowledges the warm and kind collaboration of Professor Frank Kansiime (Deputy-Principal of Makerere University College of Agricultural and Environmental sciences) who facilitated administration issues related to field sampling.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 548260, 7 pages
doi:10.1155/2012/548260

Research Article

Edge Effects on Community and Social Structure of Northern Temperate Deciduous Forest Ants

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Received 9 April 2012; Revised 13 June 2012; Accepted 27 June 2012

Academic Editor: Diana E. Wheeler

Determining how ant communities are impacted by challenges from habitat fragmentation, such as edge effects, will help us understand how ants may be used as a bioindicator taxon. To assess the impacts of edge effects upon the ant community in a northern temperate deciduous forest, we studied edge and interior sites in Jericho, VT, USA. The edges we focused upon were created by recreational trails. We censused the ants at these sites for two consecutive growing seasons using pitfall traps and litter plot excavations. We also collected nests of the most common ant species at our study sites, *Aphaenogaster rudis*, for study of colony demography. Significantly greater total numbers of ants and ant nests were found in the edge sites compared to the interior sites but rarefaction analysis showed no significant difference in species richness. *Aphaenogaster rudis* was the numerically dominant ant in the habitats sampled but had a greater relative abundance in the interior sites than in the edge sites both in pitfall and litter plot data. Queen number of *A. rudis* significantly differed between the nests collected in the edge versus the interior sites. Habitat-dependent changes in social structure of ants represent another possible indicator of ecosystem health.

1. Introduction

Data on the response of ant communities to disturbance of temperate forests in the United States are scant, despite calls to action to find indicator groups for temperate forest biodiversity conservation [1, 2]. Many of the existing studies of temperate deciduous forest ant communities in the US have been done in southern forests [3, 4]. For northern forests, studies have surveyed species diversity of ant communities, for example, for the northeastern USA [5–9], but the response of ant communities to disturbance of these north temperate deciduous forests has not been well explored. The northern forests of the eastern United States are increasingly subject to land development as 76% of northeastern forest is privately owned [10]. In Vermont, much of the forest was cleared for agriculture by the early 1800s. However, in the last century, reforestation has been extensive, as agriculture has declined, and percentage forest cover in Vermont has increased from 40% in the 1840s to 78% in 2010; the result is a landscape of highly fragmented secondary forests [11]. What impacts do that fragmentation and resultant abundance of edge habitats have on ant communities?

Habitat fragmentation, a major force decreasing biodiversity, produces landscapes with many edges, sharp boundaries between distinct patches of habitat [12]. Habitat fragmentation increases the amount of edge relative to the area of the interior of patches. Despite the facts that habitat fragmentation has greatly increased, worldwide, and that ecological edge effects have attracted much study by ecologists (e.g., [12]), our broader understanding of edge effects is still limited as studies have produced results that appear to be idiosyncratic based upon the differing ecological phenomena at work [13]. In eight studies of edge effects upon terrestrial invertebrates reviewed in Ries et al. [13], species richness/diversity increased in one study was unaffected in three studies and showed mixed responses at edges in four studies.

With respect to ground-dwelling insects, some studies of edge effects have demonstrated the importance of edge effects with respect to conservation and land management.

Golden and Crist [14] experimentally untangled two key issues related to habitat fragmentation: decreased patch sizes and increased edge, demonstrating that edge effects were more pronounced than effects of patch area on ground-dwelling rove beetle and ant species richness in old fields. Edges may be problematic because organisms living there are potentially exposed to variability in wind and weather conditions, invasive competitor species, and increased levels of anthropogenic disturbance [15, 16]. In areas with invasive ant species, the negative effects of habitat edge are clear as invaders are able to exploit the disturbed edges more readily than native competitor species [16–18]. Increased edge can lead to a reduction in biological diversity within a region, reviewed in Saunders et al. [19], and edge effects may pose a bigger challenge for insect populations than patch size.

Ant species, such as leaf-cutters or myrmecochorous species, that are highly reliant on particular plant species have been hypothesized to be particularly susceptible to negative edge effects. However, the results of studies addressing this question have been mixed. Falcão et al. [20] found significant restriction of dietary composition in leaf-cutter ant colonies living in human-created edges of Neotropical rainforest habitat in Brazil. But in a southern Appalachian highland deciduous forest, Mitchell et al. [4] found the myrmecochorous ant species, *Aphaenogaster rudis*, to be more common in small patches of forest with a history of human disturbance than in larger, less disturbed patches, perhaps due to the beneficial microclimate (warmer temperatures created by opening of canopy receive more penetrating sunlight) created by past disturbance. The presence of edge habitats increases microclimatic diversity and contributes to landscape-level heterogeneity, potentially promoting increased species richness, for mobile invertebrates (e.g., butterflies [21]).

Understanding the impacts of habitat edge on ant community structure could inform our use of ants as a bioindicator taxon. In the present study we asked: How do ant communities differ between edge and interior habitats within northern temperate deciduous forests? Furthermore, we examined colony demography and social structure of the ecologically dominant ant species, *Aphaenogaster rudis*, to determine whether social behavior of this important member of the ant community varied in edge versus interior habitats following the work of Herbers and Banschbach [22] which experimentally demonstrated the impact of food availability on social structure in the ant *Myrmica punctiventris*. We discuss our results making reference to the conceptual framework for edge effects provided by Ries et al. [13].

2. Methods

2.1. Study Area. We worked in deciduous, hardwood forest at the Mills Riverside Park in Jericho, VT, USA (44°30′N, 72°66′W; sites ranging from 244 to 410 m elevation). The Mills Riverside Park is a multiple-use conservation area with trails for hiking, mountain biking, and horseback riding winding through the forests. The park contains 66.4 hectares of conserved forest. It is adjacent to privately owned land that is currently forested, and next to a busy highway, agricultural land and the Browns River. The upper reaches of the park (northern hardwood forest type) contain a stand of American beech important for the local black bear population. All of the forest is secondary, but a small number of older "witness" trees (>100 years old) remain, spared from logging for use as markers of property boundaries [23]. Since the recreational use of this island of forest is heavy, edge habitats (adjacent to trails) are subject to regular human disturbance as well as physical edge effects.

During May through July of 2003 and in the same months of 2004, we censused ants and other invertebrates in three different forest types: a mixed woodlot, a sugar maple forest, and a northern hardwood forest, defined by stand analysis done by consulting foresters for the Jericho Underhill Land Trust (full description in Appendix [23]). In each forest type, we sampled in an edge and an interior location, matched for elevation ±10 m. Edge habitat was defined as a site within 30 m of a recreational trail; in two edge sites, the trails creating the edge also delineated a park boundary. All edge sites included in this study were in forested areas of the park and edges separated patches of forested land from other patches of forested land, park-owned or privately owned. We did not work at any edges separating forested land from agricultural land, grassland or any other habitat type. Interior sites were at least 100 m from a trail or apparent habitat boundary. Since the Mills Riverside Park is a relatively small land area, centered on a hill, we used only two distance categories in relation to edge (i) within 30 meters from the edge; (ii) greater than 100 m from the edge), while matching our study sites for elevation and forest types.

2.2. Pitfall Sampling. To determine the species composition and abundance of ants in the edge and interior forest sites, we conducted pitfall trapping using centrifuge tubes (50 mL size) half-filled with a 1 : 1 Sierra antifreeze/tap water mixture as pitfall traps. In the summer of 2003, we placed 50 traps per 40 m transect along four transects, two edge and two interior, in the sugar maple forest of the park. The transects were aligned parallel to the edge, within the 30 m zone without being so close to the trail as to be subject to human disturbance of the traps. Individual traps were arranged in squares around points on the transect, with one trap centered on a point on the transect itself and the four others at each corner of a square centered on that point but one meter diagonally away from the point. We collected the 200 traps three times, at two-week intervals, for a total of 600 trap collections during the summer of 2003. During the summer of 2004, we sampled in all three forest types of the park: mixed woodlot, sugar maple, and northern hardwood. We placed two 40 m transects in each of the three forest types, with 25 traps per transect, one transect in the interior and one at the edge of each of the three forest habitats. Again, transects were aligned parallel to the edge and traps were arranged in large squares centered on the transect, as described above. We collected the 150 traps two times, at two-week intervals, for a total of 300 trap collections, in 2004. All ants collected in traps were identified to species

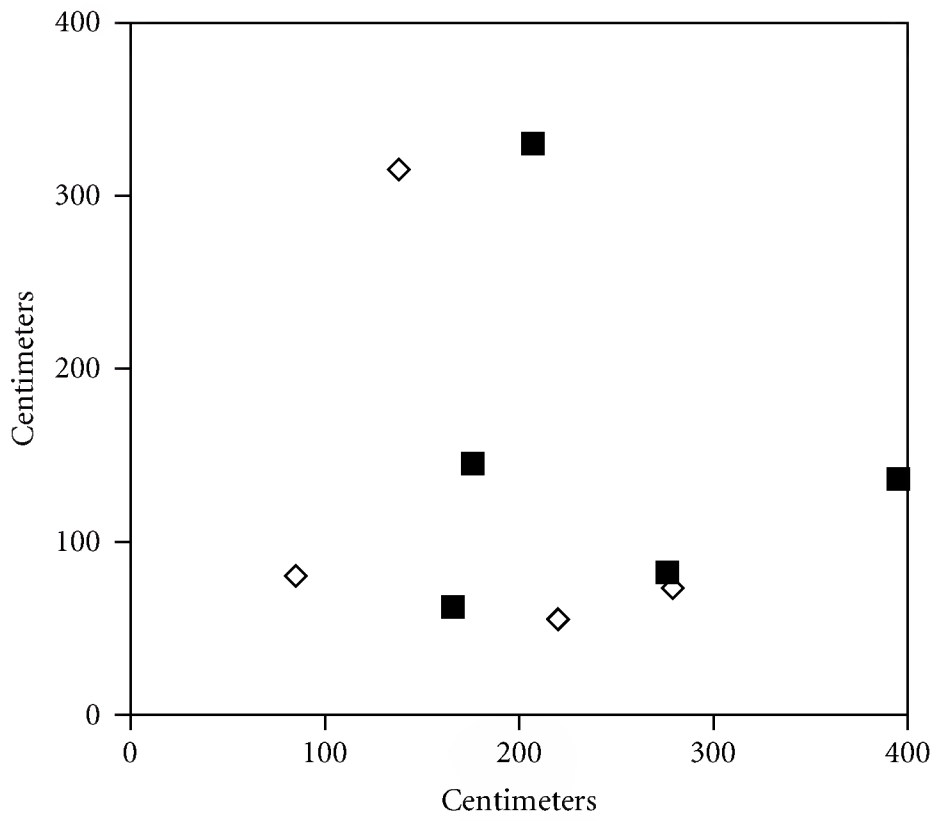

FIGURE 1: Example litter plot excavated in edge habitat. Each symbol represents an ant nest (defined as consisting of at least two workers and some brood) that was found. Filled squares represent *Aphaenogaster rudis* nests and open diamonds denote nests of *Stenamma diecki*. Individual ants walking through the leaf litter were not mapped.

with verification by S. Cover, Curatorial Assistant, Museum of Comparative Zoology, Harvard University.

2.3. Litter Plot Sampling. We excavated litter plots to estimate abundance of ant colonies to supplement the measure of individual ant abundance and diversity provided by pitfall sampling [7]. Furthermore, litter plot sampling allowed us to collect ant nests for the study of colony social structure, as in [24, 25]. To excavate litter plots, we marked off 4×4 m square plots of forest and searched the leaf litter for ant nests. We sampled one 16 m^2 plot in the interior and at the edge of each of the three forest types, mixed woodlot, sugar maple, and northern hardwood, during early June of 2004. Because of the high abundance of ants in the northern hardwood forest plots, we excavated two additional plots in that forest habitat: one plot was located in the interior and one was located at the edge, in mid-June of 2004. In total, eight 16 m^2 plots were surveyed. When nests were found, we mapped their locations on the plots (e.g., Figure 1). We collected nests of ants found in preformed cavities such as acorns, beech nuts, logs, and hollow sticks for laboratory censuses.

2.4. Colony Demography. To examine colony social structure of the most common ant species in both habitats, *Aphaenogaster rudis*, we transported nests excavated from litter plots in 2004 to the laboratory in plastic bags, and housed colonies in plastic boxes, providing glass tubing for nesting material. These colonies were maintained in the laboratory on a standard ant diet [26] and dead fruit flies. We censused the number of queens, workers, eggs, larvae, pupae, and alates in these colonies within a week of field collection.

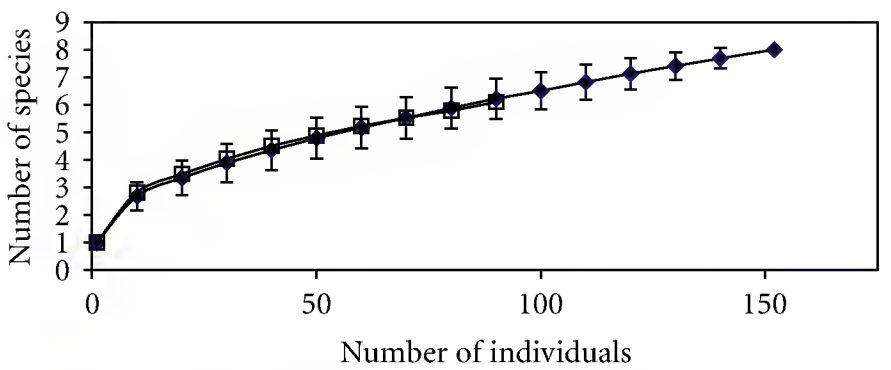

FIGURE 2: Rarefaction analysis. Expected species richness for given numbers of individuals collected in each habitat type, generated using EcoSim software [28]. Filled diamonds represent edge habitat, while open squares are for interior habitat. The 95% confidence intervals shown were generated using the edge site data.

3. Results

3.1. Species Composition and Abundance. We found 10 species of ants in the Mills Riverside Park forest sites (Table 1); pitfall samples contained 9 of the 10 species found, while litter plot excavations produced nests of 5 species including 1 species not found in pitfall traps. Nine ant species were collected in the edge sites, 7 in the interior sites. Significantly more individual ants were found in edge site pitfall traps than in interior traps (152 versus 90 individuals; $\chi^2 = 16.4$, df = 1, $P < 0.0001$), and significantly more ant nests were found in edge site litter plots than on interior site litter plots (61 versus 25 nests; $\chi^2 = 15.6$, df = 1, $P = 0.0001$) (Table 1). *Aphaenogaster rudis* (morphological form of the *Aphaenogaster fulva-rudis-texana* complex delineated by Umphrey [27]) was the most common ant overall, in terms of both frequency in samples and relative abundance (Table 1). However, the relative abundance of *Aphaenogaster rudis* was higher in interior forest habitats than in edge habitats in both pitfall data and litter plot data (Table 1).

Rarefaction analysis via simulation performed using EcoSim software [28] showed that edge and interior sites did not differ substantially in terms of ant species richness (Figure 2). The rarefaction curves in Figure 2 show the expected species richness for a given number of randomly sampled individuals in a simulation based upon our data, as described in [29, 30]. The rarefaction curves for the interior and edge sites were very similar; the interior curve lies well within the 95% confidence intervals for the edge data.

3.2. Colony Demography of Aphaenogaster rudis. Queen state of *A. rudis* nests collected from litter plots in 2004 significantly differed depending upon whether the nests were from edge or interior sites (Figure 3; Mann-Whitney U test statistic, $W = 216$, $N = 12; 13$, $P < 0.0035$); most edge nests contained a single queen, while most interior nests were queenless. Worker numbers in *A. rudis* nests from the edge sites compared to those from the interior were not significantly different (Mann-Whitney U test, $W = 185.0$, $N = 12; 13$, $P = 0.399$).

Table 1: Species composition, frequency, and relative abundance of ants. Frequency is the proportion of samples that contained individuals (in pitfall traps) or nests (on litter plots) of the species. Relative abundance is the proportion of individuals (in pitfall traps) or nests (on litter plots) of the total individuals (n) or nests collected (n). Species that were present in at least 20% of the plots or pitfall samples at either site are highlighted in bold type. In 2003, sampling was conducted on 3 dates, 1 site per date. In 2004, sampling was conducted on 2 dates, 3 sites per date.

Ant species	Interior forest sites				Edge forest sites			
	Pitfall Freq.	Pitfall Rel. Ab.	Plot Freq.	Plot Rel. Ab.	Pitfall Freq.	Pitfall Rel. Ab.	Plot Freq.	Plot Rel. Ab.
Sample sizes	450 traps	90 ants	4 plots	25 nests	450 traps	152 ants	4 plots	61 nests
Aphaenogaster rudis	**0.44**	**0.38**	**0.75**	**0.56**	**0.67**	**0.28**	**0.75**	**0.21**
Camponotus herculeanus	0.00	0.00	0.00	0.00	0.11	0.01	0.00	0.00
Camponotus nearcticus	0.00	0.00	0.00	0.00	0.11	0.01	0.00	0.00
Camponotus noveboracensis	0.00	0.00	0.00	0.00	0.11	0.01	0.00	0.00
Camponotus pennsylvanicus	**0.22**	**0.02**	**0.00**	**0.00**	**0.11**	**0.64**	**0.00**	**0.00**
Lasius alienus	**0.22**	**0.52**	**0.25**	**0.12**	**0.22**	**0.04**	**0.25**	**0.07**
Lasius nearcticus	0.11	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Myrmica punctiventris	**0.22**	**0.01**	**0.00**	**0.00**	**0.22**	**0.01**	**0.25**	**0.02**
Stenamma diecki	**0.22**	**0.04**	**0.50**	**0.12**	**0.11**	**0.01**	**0.50**	**0.57**
Temnothorax longispinosus	**0.00**	**0.00**	**0.50**	**0.20**	**0.00**	**0.00**	**0.50**	**0.13**

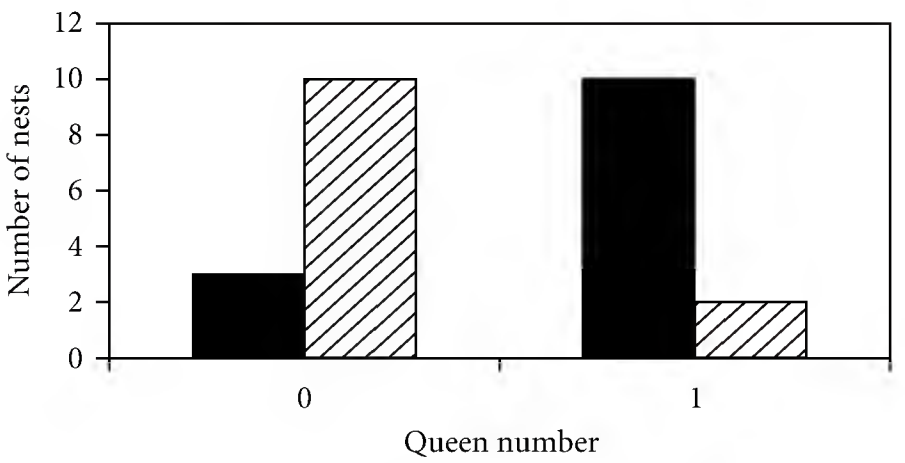

Figure 3: Queen number in *Aphaenogaster rudis* nests. Nests were collected in edge (solid bars) and interior habitat litter plots (diagonal hatched bars). Sample sizes were $n = 13$ edge nests and $n = 12$ interior nests.

4. Discussion

The edges we investigated were ones presenting a steep gradient in the main environmental variable (forest cover) defining the boundary, as the edges were created by the trails running through the park, forming linear gaps in the forest cover. The trails were mostly only two to three meters wide, but the traffic and maintenance activities associated with the trails create a much broader zone of disturbance. Given the size scale that is relevant to ants, we predicted that these edges would be meaningful enough to be impactful, despite the fact that the larger habitat patches on either side of the edge zone consisted of quite similar forest types. We found that ant species richness was not significantly different between our edge and interior sites, but abundance of individual ants and ant nests differed significantly, with more individual ants and ant nests in edge habitat.

Ries et al. [13] developed a predictive model of edge effects based upon ecological flows of light, heat, moisture, wind, as well as species interactions, species distribution and resource distribution. The Ries et al. [13] model predicts negative impacts of edge when edges separate a higher-quality habitat from one that is degraded or of lower resource quality, positive impacts of edges when edges separate patches that provide complementary resources or when edge habitat leads to a concentration of resources, and neutral impacts of edge when the edges separate patches of similar resource levels. In our case, the edges studied separate larger patches of similar resource levels (forested land of similar specific forest types) but also created a small zone of highly concentrated resources (e.g., dead wood leftover from trail maintenance activities).

Our results may be in accord with the Ries et al. [13] model in two ways. First, we saw no impact on ant species richness, likely due to the fact that our edges separated larger patches of similar resources with similar species richness. Second, our finding that abundance of both individual ants and ant nests were greater in edge habitats also is in accord with the idea that concentrated resources at the edges would have a positive impact, particularly for species that can utilize the particular resources predominant at edges [13]. Although we did not quantitatively characterize the habitat features, an abundance of downed wood was an obvious resource difference at edges compared to interior habitats (Banschbach, pers. obs.) that would have ramifications for ant abundance.

The dominant ant species at our study site is the myrmecochorous ant *Aphaenogaster rudis*. Other ant species appeared more frequently in the edge (e.g., *Camponotus pennsylvanicus*) decreasing the relative abundance of *A. rudis* in the edge, but the frequency of *A. rudis* was highest in edge habitat. One important kind of food for *A. rudis* is

the seeds of perennial herbs [31–33]. Ness and Morin [32] suggest that in edge forest habitats seed-eating rodents are more prevalent, out competing *A. rudis* for that food source, leading to a habitat preference for interior versus edge plots but we did not find clear support for that as the frequency of *A. rudis* workers in edge habitats was greater than in interior habitats. Our results are more similar to those of Mitchell et al. [4] who found both *A. rudis* and *Camponotus* spp. to be more frequent at baits in smaller habitat patches (which would have greater edge to volume ratios than larger patches) in southern Appalachian highland temperate forests.

We employed both pitfall trapping and litter plot excavations to census the ant community. Mitchell et al. [4] and Ness and Morin [32] relied upon bait attendance to census ant communities. Pitfall trapping provides an estimate of ant diversity and abundance but can be biased by the nonrandom movement patterns of individual ants and the patchy distribution of ant nest sites [34, 35]. Nevertheless, in an assessment of the efficacy of different sampling methods for assessing ant species richness and community structure, Tista and Fielder [36] concluded that pitfall trapping produced the greatest species numbers in temperate montane and floodplain European sites. Our litter plot results were in accord with our pitfall data, but the wide ranging, large individual, and colony-sized *Camponotus* spp. was found only in pitfall traps and not as nests on litter plots (Table 1). Furthermore, we collected more of the small, preformed cavity (e.g., acorn) nesting species *Temnothorax longispinosus* via nests we excavated from litter plots rather than by individual ants falling into pitfall traps (Table 1). With such low ant species richness overall, multiple methods were important to use.

The ant species richness in our pitfall trapping and litter plot collections was low (10 total species) but in keeping with other surveys of the Vermont ant fauna in second-growth hardwood forests [5, 7, 37]. Additional data we collected using food baits did not add any species to the sample (Yeamans, unpublished data). Nevertheless, greater ant species richness has been found in other habitats in Vermont such as sandplain forest [38] and lowland forests adjacent to bogs [6]. Jenkins et al. [39] found temperature to be of key importance in predicting ant species density globally. The climate in Vermont is a northern temperate climate, with an average minimum temperature of 1.8 degrees Celsius in Burlington, VT, the nearest weather monitoring station to our study site (http://www.erh.noaa.gov/btv/climo/BTV/monthly_totals/avgmin.shtml) and a very short active season for ants. Majer et al. [40] suggest that one reason for the paucity of studies on the use of ants as bioindicators in the temperate regions of Europe and North America is the relatively low species richness of ants in these world regions. For using ants as an indicator taxon for biodiversity in this habitat (using as surrogates for diversity across other taxa, McGeoch [41]), it would be challenging to correlate ant species richness with that of other taxa given the low overall richness for the ants.

Since *Aphaenogaster rudis* is such a dominant ant in our study sites and throughout temperate deciduous forests in the USA, our data regarding differences in social structure in edge versus interior habitats have some interesting possible ramifications. We found that almost none of the nests we excavated from litter plots in the interior forest contained queens, while the reverse was true of the nests we collected in the edge habitats of the forest. Since we excavated litter plots down to bare earth, we are certain that we removed all ants and brood present on these plots and did not leave any queens behind. *A. rudis* has been described as a monogynous ant species [31, 42], but other details of colony social structure and reproduction are less certain [43]. The Mediterranean *Aphaenogaster senilis* is a related monogynous species that reproduces exclusively by colony fission [44]. If this colony fission process is the main reproductive means for *A. rudis*, then the lack of queens found in the nests in our interior forest plots could be attributed to fission events occurring in advance of the production of new gynes. Boulay et al. [45] experimented with *A. senilis* and demonstrated with microsatellite analysis that after the reproductive season, many colonies were headed by a young queen who was not the mother of the workers in the colony. Since we censused ants prior to the alate production season in Vermont (late summer, early Fall), we may have detected the early evidence of the colony fission events. Resources for *A. rudis* may be more plentiful in the interior than the edge, at least in terms of reduced competition with rodents for seeds of perennial herbs [32], or perhaps general foraging competition with *Camponotus* spp.; therefore, colonies were able to fission sooner, having acquired the resources necessary to reach sufficient size to do so. Boulay et al. [45] found that *A. senilis* colonies adjust the timing of their fission events in response to competition and, therefore, resource availability.

Many studies of social structure in other ant species have documented the importance of resource availability to queen number in temperate or boreal forest ants (*Formica* spp., [46]; *Myrmica punctiventris*, [22]; *Temnothorax longispinosus* [47]). We think that future study of the genetic structure of *Aphaenogaster rudis* colonies in different habitat types could provide support for the idea that edge habitats disadvantage *A. rudis* colonies, delaying the time to fission. Because of *A. rudis*' important role as a disperser of perennial herb seeds [33], this edge effect has ramifications for the forest plant community and the forest ecosystem overall. More generally, further studies of edge effects that quantify resource availability in relation to patterns of ant diversity and abundance could substantiate the idea that edges creating a concentration of certain resources lead to increased abundance of species utilizing those particular resources.

Appendix

Description of our forest habitats in the Mills Riverside Park. This information was taken from the forest stand analysis generated by Kara Wires and Scott Moreau for the Jericho Land Trust, in 2000 [23].

Mixed Woodlot. 38% red maple, 16% white pine, 16% red spruce, 13% sugar maple, 12% black cherry and fewer ash,

aspen, paper birch and yellow birch. The approximate stand age ranged from 30 to 70 years, at the time of our study. Elevation ranges from 220 m to 280 m.

Sugar Maple Forest. 64% sugar maple and fewer ash, paper birch, red maple, red oak, red spruce, aspen, yellow birch, beech, and hop hornbeam. The approximate stand age ranged from 60 to 80 years, at the time of our study. Elevation ranges from 287 m to 384 m.

Northern Hardwood Forest. 60% sugar maple, 21% yellow birch, 16% red maple, and fewer beech, paper birch, and red spruce. The approximate stand age ranged from 30 to 70 years, at the time of our study. Elevation ranges from 299 m to 408 m.

Acknowledgments

This work was supported by a Vermont Genetics Network grant to V. S. Banschbach. The Vermont Genetics Network funding was through Grant (no.) P20 RR16462 from the BRIN program of the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH). The contents of this paper are solely the responsibility of the authors and do not necessarily represent the official views of NCRR or NIH. A. Brunelle, A. Gulka, and M. Holmes were each supported by a Vermont Genetics Network Student Summer Fellowship. R. Yeamans was supported by a Clare Boothe Luce Fellowship and a Lake Champlain Research Consortium Research Grant. The authors would also like to thank Livy Strong and the Jericho Underhill Land Trust for allowing them to work in the Mills Riverside Park and for providing them with the management plan and the forestry stand analysis for the Park. And, they appreciate the thorough reviewer comments that allowed them to substantially improve this paper.

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Hindawi Publishing Corporation
Psyche
Volume 2012, Article ID 231638, 7 pages
doi:10.1155/2012/231638

Research Article

Efficiency of Buzzing Bees in Fruit Set and Seed Set of *Solanum violaceum* in Sri Lanka

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Received 16 January 2012; Revised 3 June 2012; Accepted 12 June 2012

Academic Editor: Zachary Huang

Plant-pollinator interactions are often considered as tightly coevolved, mutualistic relationships. The present study aimed at determining the flower visiting bees of the vegetable crop, *Solanum violaceum*, and the efficiency of buzz pollination by bees on fruit and seed production in Sri Lanka. Seven bee species: *Hoplonomia westwoodi*, *Amegilla comberi*, *Patellapis kaluterae*, *Xylocopa tenuiscapa*, *Apis dorsata*, *Trigona iridipennis*, and *Ceratina hieroglyphica* visited the flowers of *S. violaceum*, and the first four species were buzzing bees. Buzzing bees were the first to visit *Solanum* flowers and were followed by nonbuzzing bees. Handling time of *H. westwoodi* and *P. kaluterae* varied with the availability of pollen in anthers that deplete with the age of flower and stayed longer at new flowers than at old flowers. Handling time of the larger buzzing bee, *H. westwoodi*, was higher than that of the smaller *P. kaluterae*. The fruit set, seed set, and seed germinability in flowers visited by buzzing bees were significantly higher than those of the flowers bagged to exclude pollinators.

1. Introduction

Plant-pollinator interactions are very complex [1] and nearly three-quarters of Angiosperms rely on animal vectors to move pollen among flowers [2]. Colour, shape, and odour are well-known characteristics of flowers which partly determine the types of animal pollinators that visit them [3]. Unlike the majority of flowering plants in which the anthers open by splitting along the entire locule, many unrelated plants displays an unusual anther rupture mechanism in which certain anthers are poricidally dehiscent [4]. Anthers of 72 families and 574 genera of the flowering plants species dehisce via pores and of them 54 families and 357 genera restrict pollen removal by buzzing bees [5]. Pollen removal requires bees that land on the flowers [6], curl around the "anther cone" [4], and vibrate their indirect flight muscles at high frequency in contact with anthers and thereby induce rapid pollen liberation [7]. This produces an audible buzzing sound and is a unique form of pollination termed "buzz pollination" [5].

Many bees including solitary and social species, and both generalists and specialists routinely use sonication to harvest pollen [8]. Sonicating bees are found in most of the major bee families in the world, in at least seven families and over 50 genera [9]. Buchmann and Cane [8] further stated that the two genera, *Apis* and *Trigona* have never been observed to sonicate poricidal flowers. Bees belonging to the genera *Hoplonomia, Lasioglossum*, *Patellapis* (family Halictidae), and *Amegilla* and *Xylocopa* (family Apidae) have been observed to buzz at flowers with poricidal anthers in Sri Lanka [10].

Buzzing bees are usually active in early morning because anther dehiscence of most buzz flowers occurs during this period. The total time of vibration of anthers of a given flower by a buzzing bee is termed the handling time of the bee [5]. The handling time differs from one bee species to another [11]. Buchmann and Cane [8] found a positive relationship between pollen availability and handling time for individual floral visits, indicating immediate assessment of pollen returns by bees in flowers of *Solanum elaeagnifolium*. Furthermore, they have observed bees selectively visiting younger pollen-rich flowers than older flowers spending more time on younger flowers. Solanaceae, Melastomataceae, Bixaceae, Cochlospermaceae, Fabaceae, and Dilleniaceae are

few examples of plant families that are pollinated by buzzing bees [12].

The dehiscence of anthers through two small apical pores is a feature found in many species of Solanaceae and especially in the genus *Solanum* [13]. *Solanum* flowers provide a relatively rich pollen source for bees that visit them [14]. Although they lack nectar and restrict access to pollen (having only terminal anther pores), they are heavily visited by a large number of individuals of at least a few species of bees [15]. *Solanum* is a cosmopolitan genus of more than 2000 species and is the second largest genus of flowering plants [8]. The genus *Solanum* is of worldwide economic importance, including major crop species such as *Solanum melongena* (eggplant) and *S. tuberosum* (potato). Even if the general syndrome of *Solanum* pollination is well known, there is little information [11, 16] about specific pollinators and pollination of *Solanum* species [15]. *Solanum violaceum* is a delicate perennial species often cultivated as an annual shrub. *Solanum violaceum* is considered as a wild plant in most of the world, but is a vegetable crop in Sri Lanka. However, the fruits are mostly collected from wildly grown shrubs and are very expensive in the local market. No studies have been conducted so far to identify the wild pollinators of *S. violaceum* and their importance in fruit and seed set. Therefore, the present study was designed to (i) determine the time of stigma receptivity and anther dehiscence in *S. violaceum* flowers, (ii) identify the bees that collect and carry pollen of *S. violaceum* and record their activity period, (iii) investigate the handling time of different bee species at *S. violaceum* flowers at different age (during peak activity period of bees), and to (iv) assess the fruit set and seed set in bagged flowers to prevent insect visits and open flowers that receive insect visitors.

2. Materials and Methods

The study was conducted in two sites: one located in Meewatura; Agriculture Research Field 7°15′N, 80°45′E) in the Peradeniya University Park in the Kandy district and the other in a home garden (7°15′11″N, 80°21′2″E) in the Kegalle district. Pollination trials were conducted only in the field at Kegalle where 25 seedlings were cultivated for the experiment.

2.1. Determination of the Time of Stigma Receptivity and Anther Dehiscence. Time of stigma receptivity and anther dehiscence was observed in freshly opened five flowers of *S. violaceum*. The time of stigma receptivity was investigated by observing the stigma through a hand lens at every 10 minutes from 6.30 a.m. to 2.00 p.m. The stigma was touched by a needle tip to observe the stickiness and was considered as the time of stigma receptivity. The time of anther dehiscence was observed by shaking the flower onto a white paper every 10 minutes from 6.30 a.m. The time at which pollen was released and collected onto the white paper was considered as the time of anther dehiscence.

2.2. Determining the Number of Pollen Grains in Anthers of Flowers at Different Age. Flowers at different age, new, 1-day-old, 2-day-old and >2-day-old flowers were selected. From each flower, one anther was removed and placed in a solid watch glass. The anther was dissected longitudinally and the pollen grains were removed into the watch glass. One milliliter of 50% alcohol was added into the solid watch glass containing the pollen grains. From this mixture, 1.00 ml volume was transferred on to a Sedgewick-Rafter Cell (a hemocytometer). Five cells with high amount of pollen grains were counted and the average number of pollen grains in the chamber was estimated. This was repeated for flowers at different age using five anthers at each age category.

2.3. Collection and Identification of Bees Visiting S. violaceum Flowers. Bees visiting flowers of *S. violaceum* were collected using a sweep net. At the Kegalle site, flower visiting bees were observed for ten sunny days until no new species were recorded. Buzzing bees were identified by the audible buzzing sound they produce at anthers during pollen gathering from anthers. Bees that do not produce an audible sound at anthers were grouped as nonbuzzing bees. Bees were collected from May 2009 at Meewature site where the preliminary survey was conducted and from August to November 2009 at Kegalle site. Bees were identified using keys to identify bees of Sri Lanka [17] and reference collection of bees lodged at the invertebrates systematics and diversity facility (ISDF) in the Department of Zoology, University of Peradeniya.

2.4. Recording of Activity Time of Buzzing and Nonbuzzing Bees Visiting Solanum Flowers. The activity time of both buzzing and non-buzzing bee species were observed at the Kegalle site. The activity time of bees were determined by their visits to flowers between 7.00 a.m. to 4.00 p.m. on four sunny days. The abundance of each bee species was not determined.

2.5. Determining the Handling Time of Two Common Buzzing Bees on Flowers at Different Age. The most common two buzzing bees that visit *S. violaceum* flowers were selected to study their handling time. Twenty flower buds ready to open were selected at the Kegalle site. The total time that a particular bee species buzzed at each of the selected flowers was recorded on 30/11/2009. On the following day, these flowers were considered 1-day old and the total handling time of each bee species at these anthers was recorded. On the third day, these flowers were considered 2-days old and the total handling time of each bee species was recorded. On the fourth day, these 20 flowers were considered >2-days old and the total handling time of each bee species at flowers were recorded. Accordingly, the total handling time of the two bee species on five new, 1-day-, 2-day- and >2-day-old flowers was observed only on sunny days (to minimize the effects from changing environmental conditions) from 30/11/2009 to 03/12/2009 for *H. westwoodi,* and from 04/12/2009 to 07/12/2009 for *P. kaluterae*. Ten specimens of each of the two common female (male bees do not buzz at flowers to collect pollen) buzzing bee species were measured for body length

to investigate the difference between the body length of the two buzzing bee species.

2.6. Study of the Efficiency of Buzz Pollination by Bees for Fruit Set and Seed Set of S. violaceum. The pollination trials were conducted from August 2009 to December 2009 in the site at Kegalle to study the efficiency of buzz pollination by bees for fruit set and the seed set of *S. violaceum*. Fifteen bunches of flower buds were randomly selected and covered by fine mesh bags to prevent visits of bees to flowers. Another randomly selected fifteen bunches of flower buds were tagged and kept open for bees to visit. Average number of fruits produced, and the number of seeds in each fruit in the two treatments were counted. Seeds obtained from each fruit from the two treatments were counted and allowed to germinate on wet tissues in Petri dishes. The number of germinated and ungerminated seeds produced from the two treatments (bagged and open) was counted.

2.7. Data Analysis. Data obtained from the study were analyzed using Minitab 14.0 and MS Excel-2007. MS Excel-2007 was used to compare the difference in the number of pollen grains in anthers of flowers at different age and the variation in handling time of the two common buzzing bee species at different age of flowers. A nonparametric test (Kruskal-Wallis test) was conducted to determine whether there is a significant relationship between the number of pollen grains per anther in flowers at different age. Two-sample t-test was carried out using Minitab 14 to determine whether there is a significant difference between handling time of the two common buzzing bees at flowers of each age category and the difference between the body length of the two common buzzing bee species at 95% confidence interval. The same analysis was conducted to test the difference in start of activity and end of activity between the non-buzzing bees and buzzing bees on *S. violaceum* flowers. The number of fruits, seeds per fruit, and the number of germinated seeds per fruit produced from the two treatments (open and bagged flowers) were also analyzed using two-sample t-test.

3. Results

In the nursery, seedlings appeared within 20 to 30 days after planting of seeds of *S. violaceum*. Seedlings took 15 days to reach the planting stage and 120–130 days to reach the reproductive stage. Upon maturity, the plants produced flower buds within five to eight days and they developed into flowers within one week. Fruits were produced within 65–70 days. On average, the plants took nearly seven months to produce mature fruits starting from the seedling stage. Flower buds took nearly one week to develop into flowers. New flowers opened around 7.30 a.m. and the lifespan of a flower was three to four days. Stigma of flowers remained receptive between 8.00 and 11.30 a.m. after blooming of flowers. Anthers dehisced between 7.30 and 8.00 a.m. Pollen grains of *S. violaceum* are yellowish-white, dry, and nonsticky with 0.02 mm of length and breadth.

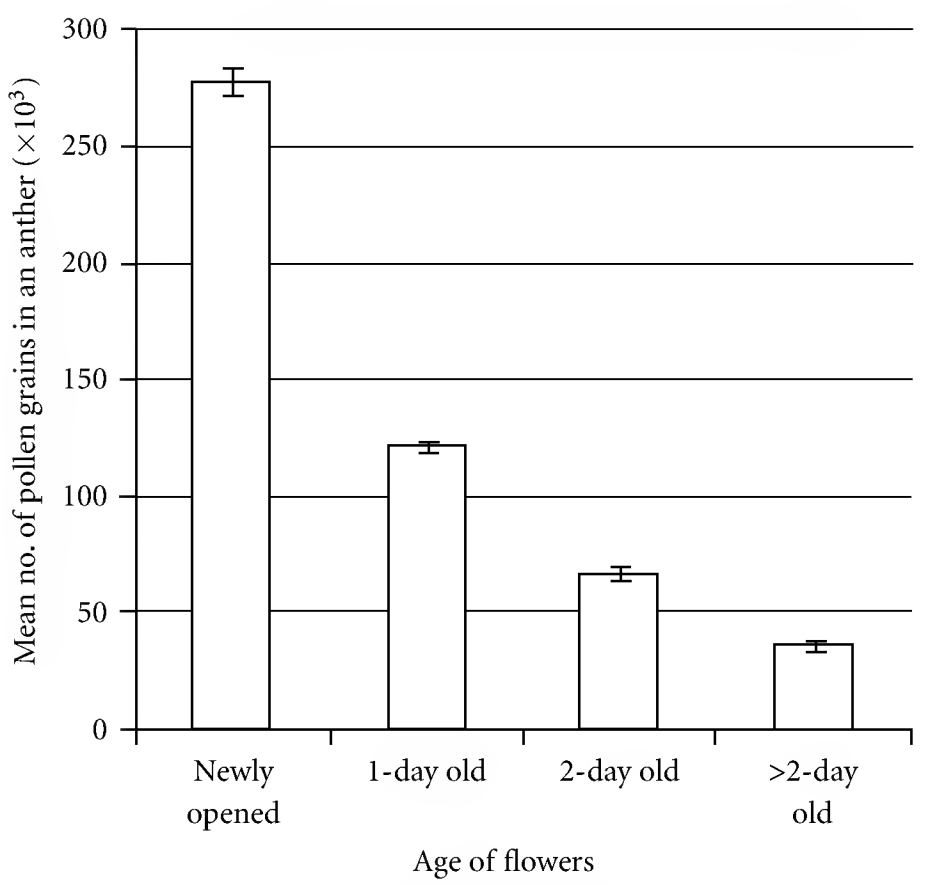

FIGURE 1: Variation in the mean number of pollen grains in an anther of *Solanum violaceum* as a function of the age of the flower.

3.1. Number of Pollen Grains in Anthers with Flower Age. Figure 1 shows the variation in the mean number of pollen grains in anthers with the age of the flower. The highest amount of pollen grains was found in new anthers. The lowest amount of pollen was found in anthers more than two days old. The results of the Kruskal-Wallis test were significant ($H = 17.97$, 3 d.f., $P = 0.000$) indicating that the mean number of pollen grains in anthers was significantly different among the different age categories of flowers.

3.2. Bees Visiting Flowers of S. violaceum. The preliminary survey conducted at Meewatura recorded four species of bees visiting flowers of *S. violaceum* of which, *Hoplonomia westwoodi* and *Patellapis kaluterae* (family Halictidae) were the buzzing bees and *Trigona iridipennis* and *Apis dorsata* (family Apidae) were the non-buzzing bees.

At the Kegalle site, *S. violaceum* flowers were visited by four species of buzzing bees namely; *Amegilla comberi*, *Xylocopa tenuiscapa* (family Apidae), *H. westwoodi* and *P. kaluterae* (family Halictidae), of which the latter two species were the most common. *Xylocopa tenuiscapa* was the rarest species and was mostly found hovering above the crop field. *Ceratina hieroglyphica*, *T. iridipennis* and *A. dorsata* (family Apidae) were the non-buzzing bees at this site. The non-buzzing bees were found collecting pollen spread over the flower petals that released due to the activities of the vibratile pollinators and they were found foraging on stigmae as well.

3.3. Activity Time of Pollen Carrying Bees on Flowers of S. violaceum. The pollen carrying bees were observed to study their activity time in flowers of *S. violaceum* on five sunny days. Activity time of buzzing and non-buzzing bees that visited *S. violaceum* flowers is given in Figure 3.

The first to visit flowers of *S. violaceum* were the buzzing bees and were followed by the honeybees. The starting time of activity between non-buzzing bees and buzzing bees was significantly different (P-value = 0.000, T-value = 4.79, DF = 25). However, the end of activity between non-buzzing bees and buzzing bees was not significantly different (P-value = 0.162, T-value = 1.45, DF = 23). The non-buzzing bees; *A. dorsata, T. iridipennis* and *C. hieroglyphica* were observed mostly after 9.30 a.m. The peak activity period during which most of the bee species were active on flowers in the Kegalle site was from 9.30 a.m. to 11.00 a.m.

3.4. Handling Time of the Two Common Buzzing Bee Species in Flowers at Different Age. *Hoplonomia westwoodi* (mean body length = 8.49 mm) and *P. kaluterae* (mean body length = 6.74 mm), of which the former bee is comparatively larger in size, were the most common buzzing bee species in the site at Kegalle. The body length of the two buzzing bee species were also significantly different (T-value = 54.44, P-value = 0.000, DF = 15). Figure 2 compares the mean handling time of the two buzzing bee species on newly opened, 1-day-old, 2-day-old and > 2-day-old flowers. The longest handling time of the two bee species was observed at new flowers while the shortest handling time was at flowers >2-days-old. There was a significant difference between the age of flower and handling time of *H. westwoodi* (H = 70.85, 3 d.f., P = 0.000) and *P. kaluterae* (H = 73.61, 3 d.f., P = 0.000).

There was a significant difference between the handling time of *H. westwoodi* and *P. kaluterae* on newly opened flowers (T-value = −7.38, P-value = 0.000, DF = 35), 1-day-old flowers (T-value = −5.23, P-value = 0.000, DF = 33), 2-day-old flowers (T-value = −4.83, P-value = 0.000, DF = 36) and more than 2-day-old flowers (T-value = −2.85, P-value = 0.009, DF = 35) at 95% confidence interval. Close observations revealed that, *P. kaluterae* vibrates each anther cone of a single flower separately, spending more time at a flower compared to *H. westwoodi* that vibrates all anther cones of a single flowers together at once.

3.5. Efficiency of Buzz Pollination in Fruit Set and Seed Set of S. violaceum

3.5.1. Fruit Set. Of the 15 flower bunches that contained about 230 flowers of *S. violaceum* kept open to facilitate bee visits, 95 fruits were formed representing 41.31% of the total flowers. The highest number of fruits (10 fruits) was obtained from one of the opened bunches which had 18 flowers (55.55%) while the lowest number of fruits (4 fruits) was obtained from the opened bunch which had 12 flowers (33.33%).

Of the other 15 flower bunches that contained about 230 flowers of *S. violaceum* kept closed by fine mesh bags to prevent bee visits, only 38 fruits were formed representing 16.52% of the total flowers. The highest number of fruits (4 fruits) was obtained from the closed bunch which had 18 flowers (22.22%) and lowest number of fruits (1 fruit) was from the closed bunch which had 14 flowers (7.14%).

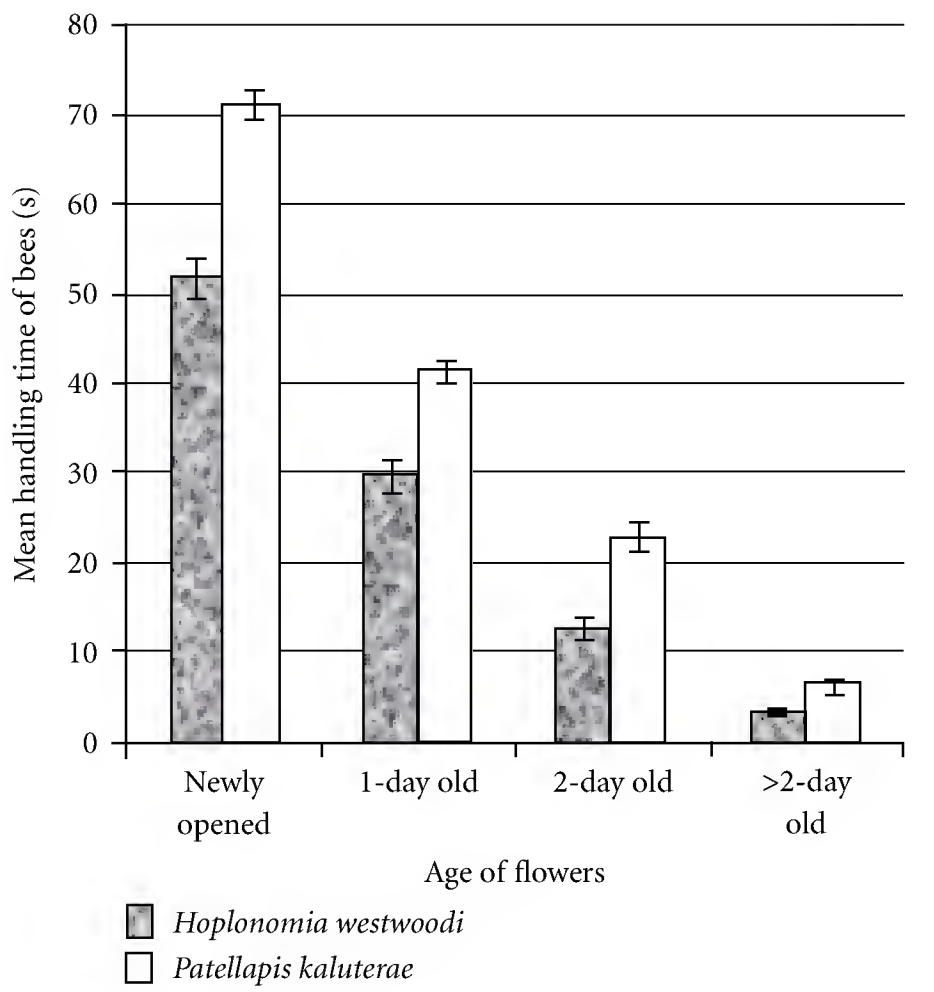

FIGURE 2: Mean handling time of *Hoplonomia westwoodi* and *Patellapis kaluterae* in *Solanum violaceum* flowers of different age in the site at Kegalle. (Mean temperature (°C), rain fall (mm), and relative humidity during the time of observations for the eight days: 30.11.2009—27.20°C, 0.0 mm, 90; 01.12.2009—26.8°C, 0.0 mm, 94; 02.12.2009—26.9°C, 0.0 mm, 88; 3.12.2009—27.0°C, 0.0 mm, 85; 04.12.2009—26.8°C, 0.00 mm, 78; 05.12.2009—26.4°C, 0.00 mm, 80; 06.12.2009—25.9°C, 0.0 mm, 79; 07.12.2009—26.1°C, 0.0 mm, 79).

Statistical analysis indicated that there is a significant difference between the number of fruits formed from open flowers and bagged flowers (T-value = −7.29, P-value = 0.000, DF = 22).

3.5.2. Seed Set. The two-Sample t-test for number of seeds formed from open flowers versus bagged flowers indicated that there is a significant difference between the number of seeds produced from open flowers and bagged flowers (T-value = 12.06; P-value = 0.000, DF = 108). Of the fruits formed from open flower bunches, 96% of the total number of 248 seeds were germinated. In fruits formed from bagged flower bunches, 92% of the total number of 208 seeds were germinated. Statistical analysis indicated that the number of germinated seeds produced from the open flowers were significantly different from that of bagged flowers (T-value = 6.34, P-value = 0.000, DF = 12).

4. Discussion

4.1. Bee Visitors of S. violaceum. As floral nectar is absent in flowers of *Solanum* [18], all the bees visited *S. violaceum* for pollen. The most common bees visiting *S. violaceum* flowers were buzzing bees belong to the family Halictidae that contains the highest number of bee species recorded for

Day	Bee sp.	7.30–8.00	8.00–8.30	8.30–9.00	9.00–9.30	9.30–10.00	10.00–10.30	10.30–11.00	11.00–11.30	11.30–12.00	12.00–12.30	12.30–1.00	1.00–1.30	1.30–2.00	2.00–2.30
28-10-2009	*H.westwoodi*		—	—	—	—	—	—	—	—	—	—			
	A. comberi				—	—	—	—							
	P. kaluterae			—	—	—	—	—							
	X. tenuiscapa														
	A. dorsata					- - -	- - -	- - -	- - -	- - -	- - -	- - -			
	T. iridipennis						- - -	- - -	- - -						
	C. hieroglyphica					- - -	- - -	- - -	- - -	- - -					
29-10-2009	*H.westwoodi*			—	—	—	—	—	—	—	—				
	A. comberi														
	P. kaluterae				—	—	—	—	—						
	X. tenuiscapa														
	A. dorsata						- - -	- - -	- - -	- - -	- - -				
	T. iridipennis				- - -	- - -	- - -	- - -	- - -						
	C. hieroglyphica				- - -	- - -	- - -	- - -	- - -	- - -	- - -				
31-10-2009	*H.westwoodi*		—	—	—	—	—	—	—	—	—				
	A. comberi				—	—	—								
	P. kaluterae			—	—	—	—	—							
	X. tenuiscapa				—	—	—	—	—						
	A. dorsata			- - -	- - -	- - -	- - -	- - -	- - -	- - -					
	T. iridipennis					- - -	- - -	- - -	- - -						
	C. hieroglyphica				- - -	- - -	- - -	- - -	- - -	- - -					
1-11-2009	*H.westwoodi*			—	—	—	—	—	—	—					
	A. comberi				—	—	—								
	P. kaluterae		—	—	—	—	—								
	X. tenuiscapa														
	A. dorsata				- - -	- - -	- - -	- - -	- - -	- - -					
	T. iridipennis					- - -	- - -	- - -	- - -						
	C. hieroglyphica														
2-11-2009	*H.westwoodi*		—	—	—	—	—	—	—	—	—				
	A. comberi														
	P. kaluterae			—	—	—	—	—	—						
	X. tenuiscapa				—	—	—	—							
	A. dorsata						- - -	- - -	- - -	- - -	- - -	- - -	- - -		
	T. iridipennis						- - -	- - -							
	C. hieroglyphica					- - -	- - -	- - -	- - -	- - -					

FIGURE 3: Activity time of seven species of bees (*Hoplonomia westwoodi*, *Amegilla comberi*, *Patellapis kaluterae*, *Xylocopa tenuiscapa*, *Apis dorsata*, *Trigona iridipennis*, and *Ceratina hieroglyphica*; — pollen bees, – – –honeybees) visiting flowers of *Solanum violaceum* from 7.30 a.m. to 2.30 p.m. during 5 sunny days in the site at Kegalle.

Sri Lanka [19]. The difference in the species composition of bees between the Kegalle site and the Kandy site indicates the site specificity of bee species visiting the same crop in different parts of the country. *Hoplonomia, Patellapis,* and *Amegilla* species are ground nesting bees [20] that cannot be reared by providing nesting places as for domesticated honeybees, leafcutter bees, and other stem nesting bees for crop pollination. This finding highlights the importance of conserving this wild bee fauna in an around crop fields even during the off season of crops. The three non-buzzing bees *Apis dorsata*, *Trigona iridipennis,* and *Ceratina hieroglyphica* may contribute to pollinate the tiny flowers of *S. violaceum* as they were found sometimes on stigmae of flowers. Anderson and Symon [15] report that *Trigona* species are very abundant on *Solanum* flowers with 99% floral fidelity and hence are significant pollinators. A similar study conducted in the Kandy site recorded *A. cerana*, the most common honeybee in Sri Lanka visiting flowers of *S. melongena* [21]. The absence of this species in *S. violaceum* flowers needs to be invesigated. An islandwide survey of insects visiting *S. violaceum* would document the different species of buzzing and non-buzzing bees in different parts of the country to reduce the biasness in results of the present study.

The buzzing bees observed during the present study are generalists that visit a wide range of flowers for pollen and nectar [10]. In flowers of *S. violaceum,* these generalist bees have become specialists to collect pollen, indicating that *S. violaceum* has restricted its pollen availability to a particular group of bees that can vibrate their anther cones to release pollen. The significant difference in the starting time of activity of buzzing and non-buzzing bees indicates the importance of the buzz pollinators to initiate pollen release that benefit the other non-buzzing bees visiting *S. violaceum*. Buzzing bees visited newly opened flowers more frequently than senescent ones with faded white petals and brown colour anthers [22, 23] and with no contrast that might provide the long-distance cue to identify the depletion

of pollen in anthers with flower age [11, 24, 25]. These signals may help the bees to spend their energy only for successful floral visits.

4.2. Age of Flower, Pollen Availability, and Handling Time of Buzz Pollinators. The release of large amounts of pollen during initial vibrations of new flowers by bees [26] decreases the amount of pollen with its age. The depletion in the number of pollen grains per anther with age of the flowers is correlated to the handling time [8, 11, 27, 28] of *H. westwoodi* and *P. kaluterae*. Harder and Barclay [12] suggested similar finding for *Solanum* flowers. The decreased handling time of the two buzzing bees, *H. westwoodi* and *P. kaluterae,* with the age of the flower might be due to the low availability of pollen which is evident from the age-dependant changes in flowers. Buchmann [5] stated an opposite finding to the present study, where the handling time is shorter at new flowers than at older flowers, as bees tend to buzz longer times in flowers with low pollen amounts. This might be possible in systems with low pollen availability, if the emerging of the buzz pollinators is seasonal and also to reduce competition among the buzzing bees active at the same time which is not similar to the present study system.

The body size of *H. westwoodi* and *P. kaluteare* inversely correlated with their handling time [15]. The difference in handling time between the two species is most likely due to their behavior in vibrating the anther cones, either singly or collectively depending on their body size. This finding is also supported by Buchmann [5] and Shelly and Villalobos [11]. According to Symon [9], only large insects are capable of buzzing and removing pollen from the anthers and function as pollen vectors. The two common bee species recorded during the present study are comparatively smaller bees. However, the largest bee in Sri Lanka, *X. tenuiscapa* that visited the flowers of *S. violaceum* that are smaller, could not handle the anther cones efficiently.

4.3. Buzz Pollination for Fruit Set and Seed Set in Solanum violaceum. The significant difference in fruit set and seed set between the two treatments may be mostly due to bee visits that facilitate removal and transport of pollen in open flowers. Buzzing bees visiting open flowers might have either cross-pollinated, self-pollinated, or might have pollinated by both methods efficiently than the closed flowers that might have self-pollinated in the absence of buzzing bees. The pollen from the cloud due to vibration by bees may directly land on stigma of the same flower facilitating self-pollination. If the bee has pollen on its body collected during previous visits from other plants, it may land on the stigma facilitating cross-pollination. Pollen from anthers of bagged flowers may release due to wind vibrations. Therefore, it seems that *S. violaceum* flowers are using both the biotic and abiotic pollinating agents for its pollination [29–32]. Close observations should be carried out to monitor bee visits to investigate the contribution of cross-pollination by bees to enhance seed set and fruit set versus self-pollination in *S. violaceum*. Kakizaki [33], Jones and Rosa [34] and Pal and Taller [35] report that *Solanum* plants grown in cages without bees produced no fruits. Baily [36] and Aizen et al. [37] report that a large number of seeds were produced by cross-pollination by bees compared to artificial pollination which resulted in fewer seeds in *S. melongena*. Fandino [24] reported that the mean number of seeds obtained from self-pollination was lower than from pollination experiments with bumblebees, showing that buzz pollination is more suitable for reaching a higher seed formation. The significant difference between the number of germinated seeds obtained per fruit produced from bagged flowers and open flowers may be due to bee pollination of open flowers that facilitates cross-pollination. In the presence of bees, germinability of *Brassica napus* has increased from 83% to 96% [38], which is also evident from the present study. The findings of the present study emphasize the importance of natural wild bees in pollinating the local naturalized exotic crops under natural conditions.

Acknowledgments

The authors are grateful to the Department of Agriculture Engineering for providing access to the Agriculture Research Fields at Meewatura, University of Peradeniya. Constructive comments made by the anonymous reviewers of the paper are gratefully acknowledged.

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