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Research Article

Evidence for Use of *Alliaria petiolata* in North America by the European Cabbage White Butterfly, *Pieris rapae*

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Pieris rapae L., an invasive crop pest, may have recently begun using *Alliaria petiolata* Bieb. (Cavara & Grande), a European invasive biennial. We investigated how *P. rapae* uses forest habitats for nectar and oviposition and examined larval performance on *A. petiolata* in the field and laboratory. Being known primarily to occupy open habitats, we found that *P. rapae* regularly uses forest edge habitats, most surveyed *A. petiolata* plants had *P. rapae* damage, and *P. rapae* successfully used both stages of *A. petiolata* for larval development.

1. Introduction

Although some of the 50,000 alien species introduced into the United States have economic value, organisms unintentionally introduced to novel habitats have been estimated to cost the United States almost $120 billion in agricultural and economic damages each year [1]. Invasive species also cause untold damages to natural habitats through changing nutrient cycles, altering resource competition, and affecting the physical landscape structure around them, including nutrient cycling [2]. Where rare species live, invasion by novel plants or animals can cause vulnerable species to become endangered or extinct [3].

Pieris rapae L. (small cabbage white; Lepidoptera: Pieridae) is a multivoltine European butterfly accidentally introduced to Quebec, Canada, in 1860. A specialist on glucosinolate-containing Brassicaceae host plants, it soon became a destructive crop pest in North America, moving south and west as far as Kentucky in just 12 years [4]. Being now ubiquitous and abundant across the United States and Canada, it is known as a butterfly of open meadows, crop plantings, and sunny areas where its cultivated and wild hosts are typically found [5, 6].

Its primary hosts in its native range include *Armoracia rusticana*, *Brassica* spp., *Cardamine* spp., *Crambe maritima*, *Sisymbrium officinale*, and *Tropaeolum majus*, among others, most of which are high light requiring plants [7]. In North America, it benefits from habitat fragmentation and disturbance favoring growth of its weedy hosts, such as *Barbarea vulgaris*, introduced *Brassica* species, and *Lepidium* species, many of which are also nonnative [8–10]. As a common pest on commercial brassicaceous crops, *P. rapae* is highly visible as an adult and more cryptic in its larval stage and has been controlled in the past through application of DDT and Bt, along with introductions of *Cotesia glomerata* and *C. rubecula* parasitoid wasps [6, 11, 12].

Although it regularly uses crop plants in North America, *P. rapae* may also use the invasive European biennial *Alliaria petiolata* Bieb. (Cavara & Grande), in part due to the plant's increasing abundance in the understory and close relationship with other host plants in the Brassicaceae. Unlike most other potential hosts, *A. petiolata* is unique in its shade-tolerance and occupancy of forest edges and understories. This invasive mustard allelopathically affects mycorrhizal forest plants as well as competes directly with neighboring plants for resources [13, 14]. Anecdotal observations suggest that this plant is much more abundant in North America than in Europe, and its presence may draw *P. rapae* into forests more often [15].

There are not many herbivores that use *A. petiolata* as a food source in North America. Although Yates and Murphy [16] identified three arthropod herbivores present on *A. petiolata* in Ontario, Canada (*Ceutorhynchus erysimi* Fabr., *Plutella xylostella* L., and *Philaenus spumarius*), they did not observe *P. rapae* consuming *A. petiolata*, and no herbivore eats enough to control its spread or abundance. Even mollusks avoid consuming *A. petiolata*, instead preferring more palatable native plants [17, 18]. This suggests that *A. petiolata* is generally well defended from most North American herbivores, and the damage that it does accrue rarely reduces plant fitness. However, *P. rapae* may be able to use the European plant as a host in North America, especially since there is some evidence of it being used as a host in Europe [7]. At present, only rare, anecdotal observations exist of the use of forested habitats by *P. rapae* in North America [19–21].

To investigate how *P. rapae* is using forested habitats and the host plant, *A. petiolata*, in North America, we directly observed *P. rapae* oviposition and nectaring behavior in forested habitats shared with *P. virginiensis*, a native congener. We also investigated how *P. rapae* uses *A. petiolata* in forest edge habitats. Finally, we compared the performance of *P. rapae* larvae and adults fed *A. petiolata* to that observed on its more typical hosts, *Brassica juncea* and *B. oleracea*.

2. Methods

2.1. Direct Observations of P. rapae in Forest Habitats. Observations of *P. rapae* occurred from April to June in 2011, 2012, and 2013 at three sites known to be occupied by *P. virginiensis*: a private site in Morrow Co. (MCO), OH, Wooster Memorial Park (WMP) in Wooster, OH, and Allegany State Park in Salamanca (ASP), NY. Basic visual observations were recorded using field notebooks and photography. Sites were surveyed in tandem with surveys for *P. virginiensis*, a related native butterfly. More details about observation protocols and site histories can be found in Davis and Cipollini 2014*a, b* publications [21, 22].

More detailed behavioral observations were made at WMP. Twenty-five *Pieris rapae* individuals were monitored between 1100 and 1600 on Apr. 15 and 18, 2012, at least 300 meters away from the nearest edge or agricultural habitat. Behaviors of individual butterflies were recorded in ten-second intervals until the butterfly left the area and included flying, gathering nectar, oviposition, and resting. Although ten seconds is longer than the time required for *P. rapae* to oviposit, we gathered enough observations to capture the rate of oviposition through time.

We identified all plants that the butterflies interacted with during oviposition and nectar gathering using the Newcomb [23] guide to wildflowers. Butterflies were identified as *P. rapae* and not as the native *P. virginiensis* by distinct, dark spots on the dorsal wing surfaces and yellow scales on the ventral wing surfaces. In contrast, *P. virginiensis* is white with occasional wing-vein shading and light spots on the wings [4].

We also observed herbivory by *P. rapae* caterpillars at WMP during the same observation periods. Although the first instar *Pieris* caterpillars are difficult to identify to species, older *P. rapae* caterpillars develop a broken yellow line along the dorsal surface and yellow spots around the spiracles; these characters are missing in native *P. virginiensis* caterpillars [4].

2.2. Herbivory by P. rapae on A. petiolata in Edge Habitats. We examined how frequently *P. rapae* uses *A. petiolata* as a larval host plant in wooded habitats by measuring end-of-year herbivory on first-year *A. petiolata* plants in maple-beech-oak forests surrounding Dayton, OH. All herbivory experiments that follow were performed in late fall, when any *P. rapae* in the area would be in diapause as pupae. This ensured that we recorded a maximum amount of damage on individual plants.

In 2011, we surveyed approximately 9000 m^2 of a recreational trail in Beavercreek, OH, between Grange Hall Road and N. Fairfield Road (BCT, western corner: 39.734756N, 84.082472W; eastern corner: 39.724096N, 84.060070W). This trail has grass and unmanaged shrubs on the southern side and a strip of second-growth forest (20–60 m forest perpendicular to the trail) on the northern side. In 2013, we returned to resurvey BCT and also surveyed two other sites: Narrows Reserve in Beavercreek, OH (NAR, located at 39.691313N, 84.030293W), and Fairborn Community Park in Fairborn, OH (FCP, located at 39.789345N, 84.009446W). Approximately 3000 m^2 and 2400 m^2 were surveyed at NAR and FCP, respectively. All three sites had parking lots, recreation trails, and forest areas. We walked the perimeter of each study area and systematically examined every rosette *A. petiolata* within 3 m of the open area. In patches with more than 10 rosettes clumped together, we randomly chose 10 plants to sample. We surveyed 99 plants at BCT in 2011. In 2013, we surveyed 136 plants at BCT, 53 plants at FCP, and 81 plants at NAR.

Plants with at least one leaf larger than 5 cm in diameter (most *A. petiolata* individuals) were surveyed for chewing damage from caterpillars (asymmetrical, smooth holes away from the leaf edge) on fully expanded leaves. Our observations of *P. rapae* damage are indirect only because the surveys were performed after *P. rapae* caterpillars had pupated for the winter. Damage was attributed primarily to *P. rapae* caterpillars for several reasons. First, caterpillar damage is distinct from other causes of damage and disease, including deer herbivory, slug herbivory, and flea beetle damage (SLD and DC, personal observations). Second, we have observed *P. rapae* caterpillars feeding on *A. petiolata* throughout the year at these locations, and *P. rapae* is the only caterpillar that we have ever observed feeding in this area, despite reports of *Plutella xylostella* as another lepidopteran herbivore on *A. petiolata* [16]. Finally, several other researchers have confirmed these observations of *P. rapae* feeding on *A. petiolata* in both Ohio and Massachusetts (John Stireman and Frances Chew, personal communications). Although some leaf tearing and disease were noted (especially the presence of a powdery mildew fungus [24]), these observations were excluded from analysis of herbivory. Each leaf on a chosen plant was scored for leaf area loss by caterpillars from 0 to 5

(undamaged, 1–20%, 21–40%, 41–60%, 61–80%, and 81–100% leaf loss). The damage rating was converted to percent leaf loss by weighing each leaf score as follows: 0 (0), 1 (0.1), 2 (0.3), 3 (0.5), 4 (0.7), and 5 (0.9). The leaf scores for each plant were averaged into a final plant score. We used the indices 1–5 because precise measurements of leaf damage in the field were not possible.

2.3. Pieris rapae Larval Performance Assay. In order to determine the suitability of *A. petiolata* as a larval host, we examined *P. rapae* larval performance on both rosette and flowering *A. petiolata* (Wright State Forest, Dayton, OH) and on two commercial brassicaceous crops, *B. juncea* and *B. oleracea* (Meijer, Inc.). *Pieris rapae* eggs (Carolina Biological Supply) were raised on either *Brassica oleracea* L. "green cabbage" (Meijer, Inc.) or flowering *A. petiolata* and allowed to emerge as adult butterflies. We used second generation butterflies because field grown rosette *A. petiolata* (used below) was too small to be useful when the shipment of eggs arrived. Between ten and fifteen adults were placed in 75 L aquaria with artificial nectar (20% sucrose : water solution on delicate task wipes until moist) and allowed to oviposit on flowering *A. petiolata*. Eggs laid by the adult butterflies were used in the following larval performance experiment. We distributed eighty neonates evenly among the four treatments below ($n = 20$ per treatment).

After hatching, second generation neonates were placed on either field-collected (June 2014) rosette *Alliaria petiolata*, flowering *A. petiolata,* commercially purchased, nonorganic *B. oleracea* (green cabbage, Meijer, Inc.), or *B. juncea* (southern giant curled mustard, Meijer, Inc.) leaves in moist filter-paper lined Petri dishes and kept in a 16 : 8 L : D incubator at room 25 deg. C. Caterpillars were kept individually to mimic the solitary nature of *P. rapae* caterpillars in the wild. Commercial plants were rinsed with distilled water before use. We chose *B. oleracea* and *B. juncea* to represent commercial hosts available to *P. rapae* in the wild. Caterpillar habitats were cleaned daily and stocked with an overabundance of host plant material to prevent starvation or time without eating. After one week of monitoring daily for survival, we took daily measurements of caterpillar mass, until they neared pupation. Pupae were weighed and placed in 75 L aquaria according to their larval host plant, with artificial nectar and an oviposition substrate (rosette *A. petiolata*). After eclosion, butterflies were allowed to mate and oviposit freely. When all butterflies died, the number of eggs and the number of females were counted to calculate the mean number of eggs laid per female, an indirect measure of fitness.

2.4. Statistical Analysis. All statistical analyses were performed in *R* [25]. We separated our field herbivory data into two sets: data from BCT alone and data from 2013 alone. These data were separated because only one site, BCT, was sampled for two years. For both datasets, we used a binomial model with a logit link function followed by Tukey's HSD test (multcomp package) to examine how the number of leaves on a plant covaried with location (2013 data, predictor) or year (BCT data, predictor) to affect the presence or absence of damaged leaves (response variable) [26]. We also examined the same datasets (2013 and BCT data) for differences in the percent leaf loss score. We removed all zeroes because we were only interested in damaged plants, log-transformed the percent leaf loss scores to meet normality assumptions, and then evaluated the data using a generalized linear model followed by Tukey's HSD post hoc testing when appropriate. Plots were constructed with the gplotspackage [27].

For the larval performance experiments, we used the Kaplan-Meier estimator for survival data (survival package) and one-way ANOVA to compare pupal mass and relative growth rate across host plants [28]. Relative growth rate (RGR) was calculated as larval mass increase (from day 7 until pupation) divided by the initial larval mass times the number of days of recorded growth. Chi-square testing followed by chi-square tests with Bonferroni correction was used to evaluate differences between the number of eggs laid per treatment.

3. Results

3.1. Direct Observations of P. rapae in Forest Habitats. At MCO and ASP, we regularly observed *P. rapae* flying in heavily wooded areas but did not observe any nectar gathering or oviposition behavior. We found an unidentified first instar caterpillar in 2012 at MCO on *A. petiolata* that could have been either *P. rapae* or *P. virginiensis*. At WMP, we observed *P. rapae* adults gathering nectar in the understory from several plant species, including *Claytonia, Phlox*, and *Viola* species, as well as from *A. petiolata* itself. We also observed 3 female *P. rapae* ovipositing on *Cardamine diphylla* and photographed an older *P. rapae* caterpillar feeding on *A. petiolata* (Figure 1). Additionally, *P. rapae* caterpillars have been observed feeding on *A. petiolata* outside of the Wright State University greenhouse (2012–1015), on *A. petiolata* in the Wright State University woods, and in residential and park areas in Fairborn and Beavercreek, OH (SLD, personal observations).

3.2. Herbivory by P. rapae on A. petiolata in Edge Habitats. Although overall percent leaf loss was low, 78.8% of plants were damaged by caterpillars in 2013, indicating that *P. rapae* commonly uses *A. petiolata* in North America. In 2013, both the number of leaves ($z = 3.475$, $P < 0.01$) and the location ($P < 0.01$) influenced the probability of plants being attacked. BCT was significantly different from NAR ($z = 2.614$, $P < 0.05$) and FCP ($z = 3.631$, $P < 0.01$), but the latter two were not significantly different from each other ($z = -2.217$, $P > 0.05$). Across years at BCT, only the number of leaves was a significant factor in the model ($z = 3.622$, $P < 0.01$), indicating no difference in plant damage between years. Evaluating the percent leaf loss score revealed similar results, with BCT being significantly different from both NAR ($z = 2.387$, $P < 0.05$) and FCP ($z = 3.697$, $P < 0.01$), but NAR and FCP were not significantly different from each other, and the number of leaves per plant was not correlated with the percent leaf loss score. The model evaluating percent

Table 1: Mean percent survival, days to pupation, pupal weight, and relative growth rate with standard error of *P. rapae* (both sexes) between four host plants ($n = 16$ per treatment). Different letters indicate significant differences ($P < 0.05$).

Treatment	Survival (%)	Days To pupation (d)	Pupal mass (g)	Relative growth rate ($g\,g^{-1}\,d^{-1}$)
A. petiolata rosette	56.25	15.33 ± 0.85	0.128 ± 0.003	0.277 ± 0.046^{ab}
A. petiolata flowering	20.00	15.33 ± 1.45	0.112 ± 0.008	0.182 ± 0.031^{b}
B. oleracea "cabbage"	68.75	16.63 ± 0.28	0.134 ± 0.004	0.294 ± 0.019^{ab}
B. juncea "mustard"	56.25	12.78 ± 0.46	0.132 ± 0.006	0.380 ± 0.044^{a}

Figure 1: Mature *P. rapae* caterpillar consuming rosette *A. petiolata* in Wooster, OH. Photo taken on May 11, 2012, by SLD.

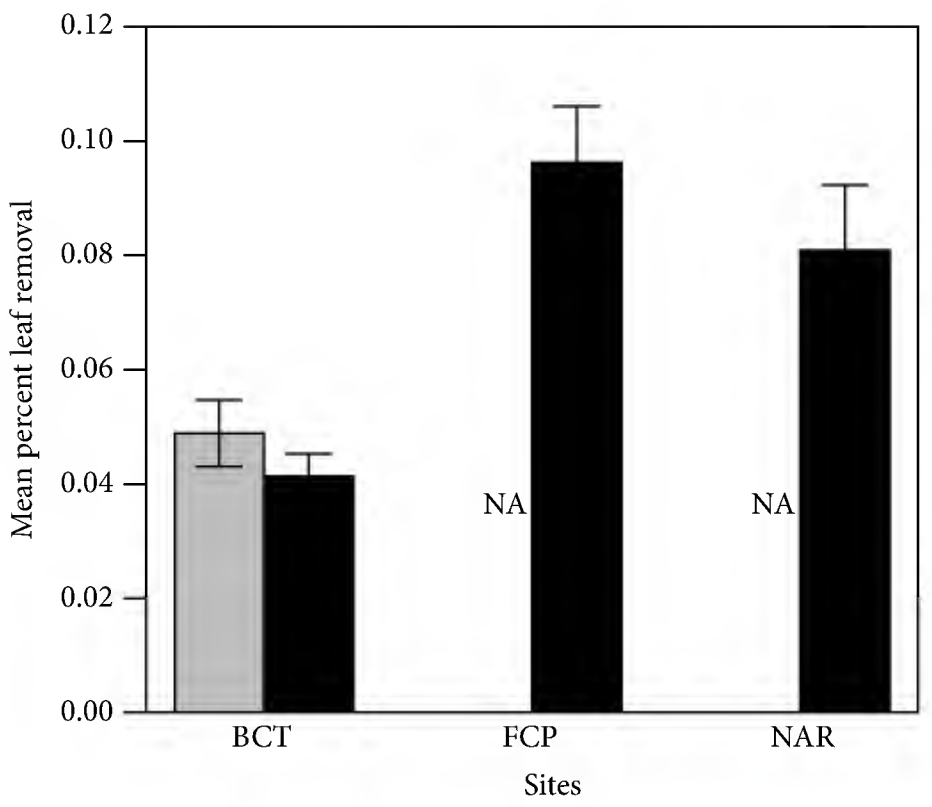

Figure 2: Herbivory (percent leaf loss) on *A. petiolata* varied between sites and habitats in 2013. Gray bar represents data from 2011; black bars represent data from 2013.

leaf loss score as influenced by date and number of leaves for the BCT site alone was not significant. Figure 2 shows the mean percent leaf loss score for both sites and years.

3.3. Pieris rapae Larval Performance. Although there was a trend towards lower survival of *P. rapae* caterpillars feeding on flowering *A. petiolata*, we found no significant differences in survival of *P. rapae* caterpillars on the four hosts that we tested ($\chi^2 = 7.4$, df = 3, and $P = 0.0596$, Figure 3).

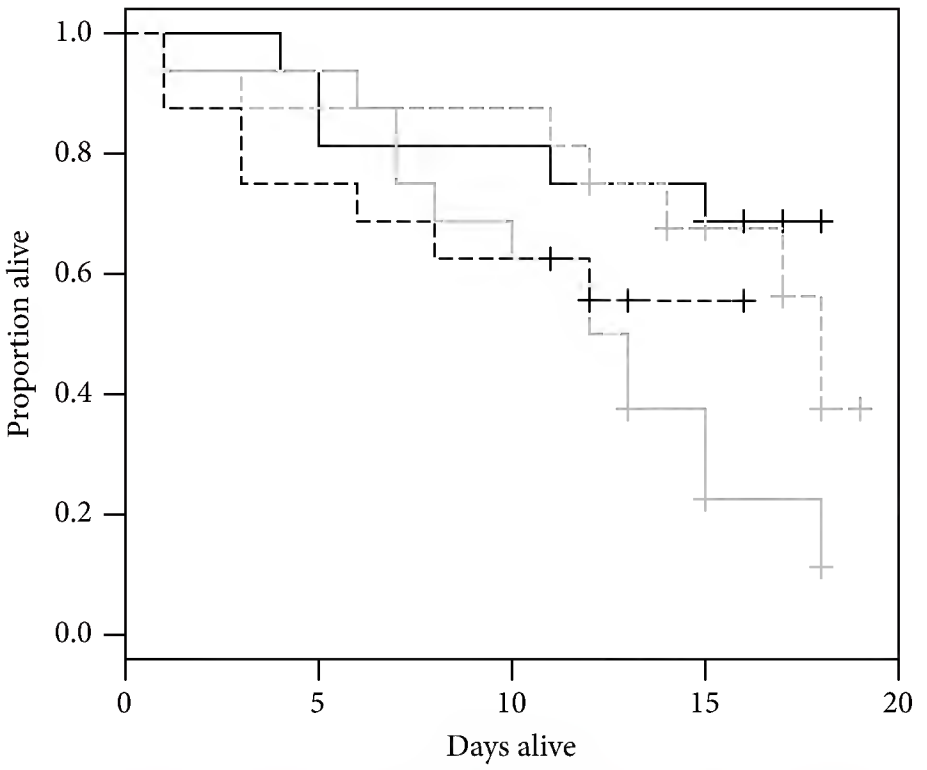

Figure 3: Kaplan-Meier survival estimates of *P. rapae* caterpillars fed commercial cabbage (solid black), rosette *A. petiolata* (dash grey), flowering *A. petiolata* (solid grey), or commercial mustard greens (dash black).

Pupal mass did not vary between treatments ($F = 2.213$, df = 3, and $P = 0.109$); however, there were differences in time to pupation ($F = 7.897$, df = 3, and $P < 0.01$). Caterpillars reared on *B. juncea* pupated significantly earlier than those raised on rosette *A. petiolata* ($P < 0.05$, Tukey's HSD) and on commercial *B. oleracea* ($P < 0.01$). Relative growth rates also differed between treatments ($F = 4.428$, df = 3, and $P < 0.01$) because caterpillars on leaves of *B. juncea* grew significantly faster than those on flowering *A. petiolata* ($P < 0.01$, Tukey's HSD). To summarize, *P. rapae* caterpillars reared on *B. juncea* grew faster and pupated earlier with no significant loss of pupal mass, whereas caterpillars reared on flowering *A. petiolata* took longer and grew slower than those on *B. juncea*. Means and standard error of each treatment are found in Table 1.

Eclosed butterflies from the larval performance experiment were allowed to freely mate and lay eggs on rosette *A. petiolata*. Butterflies raised on *B. juncea* laid 89.5 eggs per female ($n = 4$ females, 3 males), those raised on *B. oleracea* laid 176.6 eggs per female ($n = 3$ females, 3 males), those raised on rosette *A. petiolata* laid 119.5 eggs per female ($n = 2$ females, 4 males), and the lone female raised on flowering *A. petiolata* laid 147 eggs ($n = 1$ female, 1 male). A chi-square test for proportions revealed significant differences from the mean of 133 eggs per female ($\chi^2 = 31.3284$, df = 3, and $P <$

0.01). Post hoc testing showed that females laid significantly fewer eggs when raised on *B. juncea* than any of the other groups, and females raised on *B. oleracea* laid significantly more eggs than either *B. juncea* or rosette *A. petiolata* raised butterflies.

4. Discussion

We looked for evidence of the nonnative butterfly, *Pieris rapae*, using *A. petiolata* in both forest and edge habitats in North America, and also examined larval performance. Previous authors have observed occasional forest use by *P. rapae* [19–21], but we demonstrate that *P. rapae* frequents forested habitats, using both native and nonnative nectar and host plants. We also confirmed that *P. rapae* successfully uses *A. petiolata* as well as its more typical brassicaceous hosts in North America. In forests shared with *P. virginiensis*, *P. rapae* uses the same nectar and oviposition resources as *P. virginiensis*, with one exception: *P. rapae* can successfully use *A. petiolata* as a larval host, but the native congener cannot [22, 29, 30].

One possible implication regarding the use of forested habitats by *P. rapae* is direct competition for oviposition sites (and, therefore, larval food resources) by native *Pieris* species. If *P. rapae* prefers to oviposit on the primary native host of *P. virginiensis*, *Cardamine diphylla*, the caterpillars may occasionally compete for food. This competition could be limiting near pupation when native ephemeral plant hosts are in decline [31]. However, habitat sharing may benefit *P. virginiensis* if *P. virginiensis* practices egg avoidance like other congeners. If *P. rapae* prefers ovipositing on *A. petiolata* instead of on the native *C. diphylla*, *P. virginiensis* may not lay its eggs on already occupied *A. petiolata* leaves.

The presence of *P. rapae* in forests may negatively affect *adult* native pierids only if nectar is a limiting resource. Nectar resources drive Lepidopteran habitat selection and fuel successful egg maturation and oviposition [32–34]. In some cases, Lepidoptera compete directly for nectar resources, attempting to dislodge other butterflies occupying desirable flowers [35]. The initial invasion of *P. rapae* may have caused a severe decline in the abundance of another native butterfly, *P. oleracea*, before the invasion of *A. petiolata* [4], though more recent authors disagree [20]. Further work needs to be done to determine if nectar availability would be limiting for native pierids persisting in forest habitats, and whether competition for nectar with *P. rapae* is important.

In North America, *A. petiolata* is an ideal host for *P. rapae*, providing nectar each spring, as well as plant material year round (rosettes persist through winter before flowering in the spring) for larval development. Although *P. rapae* may reduce the fitness of *A. petiolata* through folivore, any fitness reduction will not be substantial. Evans and Landis [36] found that the minor foliar damage recorded in field observations of *A. petiolata* actually increased fecundity of *A. petiolata*, much like grazing can positively affect grasses. Further work needs to examine how *P. rapae* and *A. petiolata* affect each other's fitness and abundance.

In addition to its use as a larval host, the nectar resources offered by *A. petiolata* may draw more *P. rapae* to agricultural fields near forested areas and edges occupied by *A. petiolata*. Zhao et al. [37] found that *P. rapae* were more abundant in broccoli interplanted with nectar-producing plants than in broccoli monocultures. Future experiments should include an examination of *P. rapae* populations in fields with and without nearby woodlands invaded by *A. petiolata*.

There may be an increase in apparent competition for enemy-free space when *P. rapae* use forest resources in habitats already occupied by native *Pieris* species. Benson et al. [6] found no evidence that *Cotesia glomerata* L. or *C. rubecula* would attack *P. virginiensis* sentinel caterpillars near meadows; however, lab work demonstrates no preferences by these wasps for different *Pieris* spp. caterpillars as potential hosts. Despite being not currently a problem, *Cotesia* may be a problem for future generations of *P. virginiensis, P. oleracea*, and other native pierid butterflies if they begin to follow *P. rapae* into nearby forests.

Finally, *P. rapae* may interfere with volunteer-driven conservation efforts for the native *Pieris* species. There are many organizations that track *P. virginiensis* populations over time, but some volunteers estimate unusually high densities of *P. virginiensis* (C. Lehn, unpublished data). Some of these observations may be of *P. rapae* utilizing forest habitat for its nectar and oviposition resources. Differentiating between these Pierids at a distance, by sight or behavior, is difficult [20, 31]. Volunteers may be overestimating population sizes by misidentifying *P. rapae* as native *Pieris* spp. and consequently missing instances where populations are in decline or extinct.

In conclusion, *P. rapae* is present in North American forest habitats with and without cooccurring native pierid species, and its use of *A. petiolata* appears to facilitate its occupancy. *Pieris rapae* may be simultaneously escaping pressure from competition and parasitism, as well as increasing herbivorous pressure on the exotic mustard *A. petiolata*. Where *P. rapae* overlaps with native Pierids, there are opportunities for competition. However, more work needs to be done to investigate both the cause of *P. rapae* habitat expansion and the ecological implications of moving into forested habitat.

Conflict of Interests

The authors have no affiliations or involvement with any organization that has a financial interest in the results discussed in this paper.

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Research Article

New Species of *Rheotanytarsus* Thienemann and Bause (Diptera: Chironomidae: Tanytarsini) from Darjeeling–Sikkim, Himalaya, India, with Revised Keys to the Adult Males and Pupae of the Species of the Oriental Region

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Three new species of *Rheotanytarsus* Thienemann and Bause are described and illustrated from India. *R. nudicornus* n. sp. belonging to the *aquilus* species group is described as adult male and pupa, *R. spinicornus* n. sp. in the *muscicola* group is described as adult male, pupa, and larva, and *R. caputimberus* in the *trivittatus* group is described as adult male with damaged pupa. A possible placement and inclusion of these three new species from India and other seven species recorded from the Oriental China in the key to males of genus *Rheotanytarsus* of Kyerematen et al. are proposed. A probable placement and inclusion of the 2 new species from India in the key to pupae of *Rheotanytarsus* of Kyerematen et al. are also stated. Diagnoses of the *muscicola* group and *trivittatus* group are emended.

1. Introduction

The genus *Rheotanytarsus* Thienemann and Bause is a diverse predominant group occurring in nearly all lotic water recorded from all biogeographic regions except Antarctica comprising more than 100 nominal worldwide species [1]. The larvae of *Rheotanytarsus* are rheobiontic, filter feeding using nets stretching between the anterior "arms" of their characteristic cases. The silk mesh retains suspended detritus from the water flowing past the case. Detritus are utilized as food and for dwelling material by the larvae [1]. Most larvae live in moderately fast-to-slow flowing rivers, streams, and creeks and rarely in stagnant water—more likely in nearshore habitats in wind—driven currents on lakes [2]. The larvae may be phoretic on a number of other aquatic invertebrates including naiads of odonates, may flies, larvae of caddis flies, megalopteran insects, and gastropod molluscs [3].

Most species are described based on male adults including their distinctive genitalia; fewer are also known from their immature stages as Lehmann [4] described many western European species with their pupae while Cranston [5] did for Australian ones. "Composition of tentative species groups" of the genus *Rheotanytarsus* based on both pupal exuviae and adult males made by Sæther and Kyerematen [6] has been followed here. Of the three new species, *R. nudicornus* n. sp. belongs to the *aquilus* species group described as adult male and pupa, *R. spinicornus* n. sp. in the *muscicola* group as adult male, pupa, and larva, and *R. caputimberus* n. sp. in the *trivittatus* group as adult male and damaged pupa. A possible position and insertion of these three proposed Indian species and other seven species reported from Oriental China [7] including four species, namely, *R. bullus*, *R. liuae*, *R. polychaetus*, and *R. quadratus* described by Wang and Guo [7], *R. tamatertius* Sasa of Palaearctic Japan, *R. buculicaudus* Kyerematen, Andersen & Sæther of Ghana and *R. muscicola* Thienemann of Holarctic Region including Palaearctic China in the key to known males of genus *Rheotanytarsus* of Kyerematen et al. [2] are proposed. A probable placement and inclusion of the 2 new species from India in the key to known pupae of *Rheotanytarsus* of Kyerematen et

al. [2] are also provided. Moubayed described *Rheotanytarsus orientalis* [8] and *Rheotanytarsus thailandensis* [9] from Thailand. Kyerematen et al. [2] made a review of the 26 species of Oriental *Rheotanytarsus*. Chaudhuri et al. [10] recorded four species of the genus from India. Later Wang & Guo [7] reviewed the genus from China stating seven species from Oriental China. With the addition of three new species here described, the number of species now increases to seven from India and thirty-six from the Oriental Region. Diagnoses of the *muscicola* group and *trivittatus* group are emended after examination of *Rheotanytarsus spinicornus* n. sp. and *Rheotanytarsus caputimberus* n. sp., respectively.

2. Material and Methods

The larvae collected from the streams of the Darjeeling–Sikkim, Himalaya, were reared in the glass vials containing substratum of the natural habitat plugged with cotton. The specimens were mounted on microslides following the method of Hazra et al. [11]. Morphological terminologies and abbreviations follow Sæther [12] and Epler et al. [1]. Measurements are expressed in micrometers (μm) except the total length and wing length which are in millimetres (mm) with the ranges suffixed by "*n*" in parentheses denoting the number of specimens considered.

Types of the new species specimens now retained with the entomological collections of the Department of Zoology, University of Burdwan (India), will be deposited at the National Zoological Collections (NZC), Kolkata, in due course.

3. Results and Discussion

3.1. The aquilus Group [6]

Rheotanytarsus nudicornus n. sp. http://zoobank.org/NomenclaturalActs/4A09B167-16BE-43E4-ACE8-432D832DDBDF (Figures 1-2).

3.1.1. Studied Specimens. Holotype (male with pupal exuviae) (reared) (Type number B.U. Ent. 268), India, Sikkim, Jorethang (27°20′00″N; 88°35′00″E), 31/iii/1996, N. Hazra leg. Paratype (1 male), as holotype.

3.1.2. Etymology. From the Latin *nudus*, bare, and *cornus*, horn, referring to the bare thoracic horn of the pupa.

3.1.3. Description

Adult Male (n = 2) (Figure 1). Total length 2.40–2.5 mm. Wing length 1.56 mm. Total length/wing length 1.54–1.61. Wing length/length of profemur 2. Thorax light brown, abdomen and legs pale yellow.

Head. AR 0.48–0.50; flagellomere 12 (Fm 12) 192–196 μm long. Eye with 60–64 μm long dorsomedial extension. Temporal setae 6, including 2 inner verticals (IV), 2 outer verticles (OV) and 2 postoculars (Po). Clypeus with 17–19 setae. Tentorial length 105 μm, 19 μm wide at sieve pore, 9 μm wide at posterior tentorial pit. Palpomere lengths (I–V): 27 μm, 30 μm, 84 μm, 93 μm, 117 μm.

Thorax. Acrostichals 12–14; dorsocentrals 9-10; scutellars 9-10.

Wing (Figure 1(a)). Membrane covered with setae, especially in distal half. Costal length 1.59 mm. Costa not extended. CR 0.90. VR 1.51. Sc, M and Cu_1 bare, *R* with 16–18 setae; R_1 7–9; R_{4+5} 60–64; M_{1+2} 54–56; M_{3+4} 48–52; Cu 60–64; PCu 68–70; An 26–28. Cell m with 10–12 setae, r_{4+5} about 68, m_{3+4} about 10, cu and an combined about 40 setae. R_{2+3} absent. RM well proximal to FCu.

Legs. Spur of fore tibia (ti) 22–24 μm long; spurs of mid ti unequal 20–22 μm and 32–34 μm long including 36–38 of comb; of hind ti spurs 26–28 μm and 36–38 μm long including 40–42 of comb. Width at the apex of fore tibia 36–40 μm; mid tibia 32–34 μm; hind tibia 38–40 μm. Lengths and proportions of leg segments as in Table 1.

Hypopygium (Figures 1(b)–1(f)). Tergite IX with 16–18 setae, anal tergite band V-shaped, separate, not joined by basal tergite band. Anal point 40–42 μm long, 8–10 μm wide at base, 6 μm wide at apex. Crest narrowly V-shaped and basally open, 40–44 μm long. Phallapodeme 45 μm long, transverse sternapodeme 54 μm long. Superior volsella (Figure 1(d)) 45 μm long, oval with knob like little apical projection; median volsella (Figure 1(e)) relatively short, 33 μm long, subulate setae fused into plate, not extending beyond both superior and inferior volsella; inferior volsella (Figure 1(f)) 60 μm long with 11–13 setae at apex. Gonocoxite 81 μm long; gonostylus 87 μm long, 26–28 μm wide at mid point with distal part not abruptly narrowed. HR 0.93, HV 2.75.

Pupa (n = 2) (Figure 2). Total length 3.51 mm. Exuviae little dark.

Cephalothorax. Frontal apotome (Figure 2(a)) rugulose. Frontal setae 46–48 μm long, seated medially, arising from tubercles. Thoracic horn (Figure 2(b)) 216 μm long, slender, pointed at the apex and completely bare. Thorax smooth, wing sheath with prominent nose (Figure 2(c)), 18–21 μm long. Two antepronotals, one median antepronotal 36 μm long and one lateral antepronotal 18 μm long. Three precorneals, anterior one 63 μm long, lemelliform; median one 36 μm long and posterior 36 μm long. Dorsocentrals Dc_1 and Dc_2 paired 14–18 μm and 10–12 μm long respectively and Dc_3 and Dc_4 also paired, 20–24 μm and 6–10 μm long respectively; distance between two paired dorsocentrals 78–82 μm.

Abdomen (Figures 2(d)–2(g)). Tergite I bare. Tergites II–V with anterior pair of spines of circular patches. Tergites III–V with extensive shagreen present posterior of circular patches extending over and beyond the first dorsal seta, most pronounced on tergite V. Pair of circular patches on tergite V smaller than others. Median shagreen essentially absent,

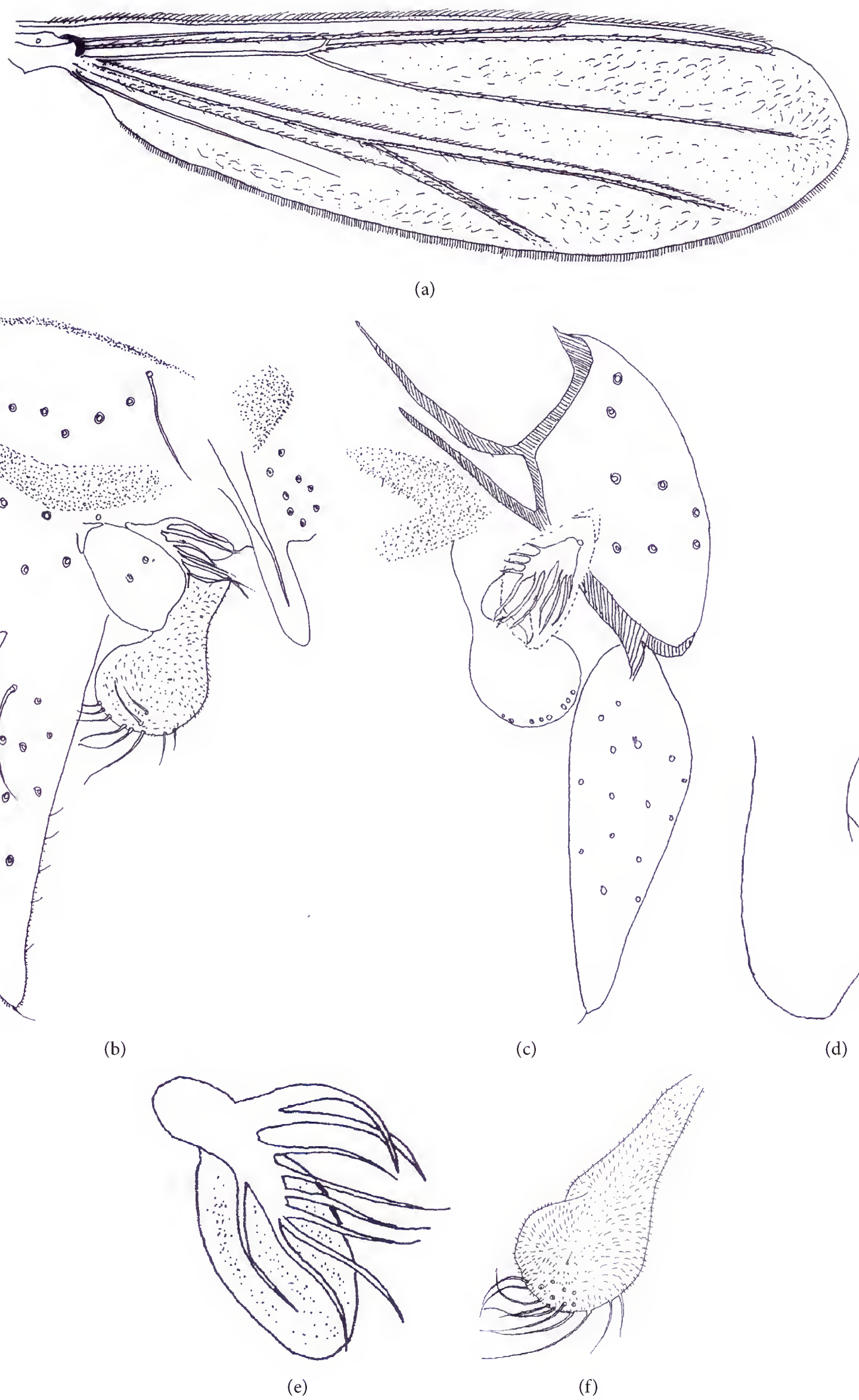


FIGURE 1: *Rheotanytarsus nudicornus* n. sp., adult male: (a) wing; (b) hypopygium (left-dorsal view); (c) hypopygium (right-ventral view); (d) superior volsella; (e) median volsella; and (f) inferior volsella.

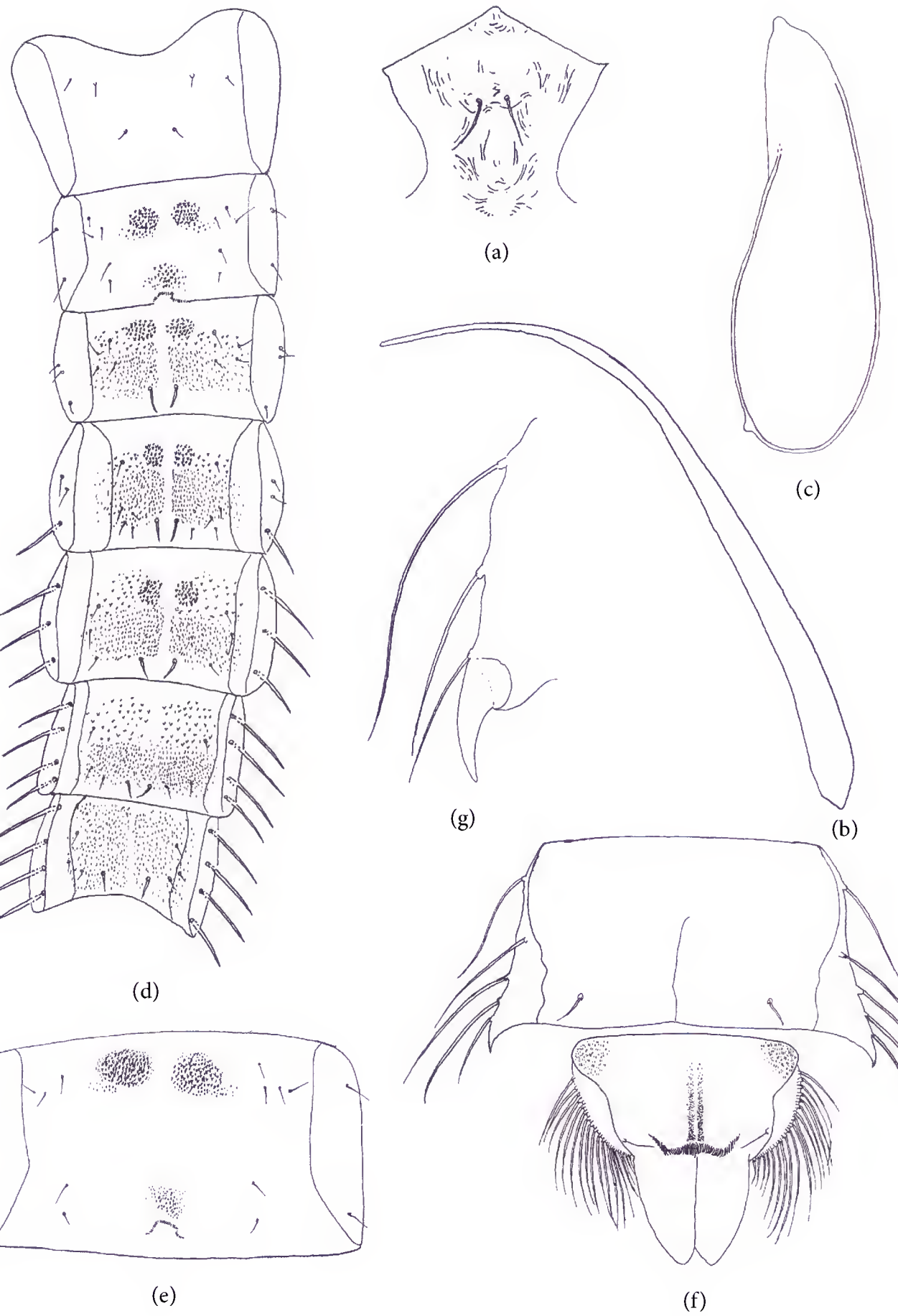


FIGURE 2: *Rheotanytarsus nudicornus* n. sp., pupa: (a) frontal apotome; (b) thoracic horn; (c) wing sheath; (d) tergites I–VII; (e) tergite II; (f) tergite VIII and anal lobe; and (g) caudolateral spur.

TABLE 1: Lengths (μm) and proportions of leg segments.

	fe	ti	ta_1	ta_2	ta_3	ta_4	ta_5	LR	BV	SV
P_1	765	405	900	465	330	420	150	2.23	1.51	1.3
P_2	675	615	345	165	120	90	75	0.56	3.64	3.74
P_3	855	705	480	285	240	135	105	0.68	2.67	3.25

weak and sparse shagreen present caudolaterally on tergites IV-V. Number of spines on patches tergites II–V: 94–100; 84–88; 70–74; 44–48. Tergite II with additional few shagreen of very fine spinules just above the hook row; hook row small, not dividing medially, occupying 0.08 μm width of the segment width, containing about 36–40 hooklets (Figure 2(e)). Conjunctives without shagreen. Segment II with 2 L setae, III with 3 fine L setae; IV with 2 L setae and 1 posterior LS seta; V with 3 LS setae; VI-VII with 4 LS setae; VIII (Figure 2(f)) with 5 LS setae. Caudolateral spur (Figure 2(g)), single, 22–25 μm long. Shagreen present anterolaterally on anal lobe, 117 μm long and 163 μm wide with 34–36 taeniae in fringe,

longest taenia 180–192 μm long. Anal lobe (Figure 2(f)) with one hair-like dorsal seta 36 μm long. ALR 1.45, G/F 1.71.

3.1.4. Remarks. Rheotanytarsus nudicornus n. sp. is close to Afrotropical species *R. aquilus* Kyerematen & Sæther [13] in number of flagellomeres, wing length, number of acrostichals, absence of basal tergite band, median volsella not reaching beyond the apex of superior volsella but differs in AR, HR, shape of median volsella, anal point and anal crest. The species may be separated from other species of the *aquilus* group including oriental species *R. bullus* Wang & Guo [7], *R. kuantanensis* Kyerematen, Andersen & Sæther [2], *R. madarihatensis* Kyerematen, Andersen & Sæther [2], and *R. polychaetus* Wang & Guo [7], with 12 flagellomeres and gonostylus not abruptly narrowed by the following combination of characters: (i) anal tergite band V-shaped not joined by basal tergite band, (ii) anal crest narrowly V-shaped and open at the base, (iii) superior volsella oval with knob-like small apical projection, (iv) subulate setae of median volsella fused to form a plate in adult male, and (v) bare thoracic horn, without a median bend, (vi) tergites II–V with anterior pair of spines of circular patches, (vii) posterior spinules on tergite II undivided, and (viii) anal lobe with hair-like dorsal seta of the pupa.

3.2. The muscicola Group [6]

Emended Diagnosis. Anal lobe of pupa with or without dorsal seta and superior volsella of male with or without knob-like or slightly hooked posterior extension.

Rheotanytarsus spinicornus n. sp. http://zoobank.org/NomenclaturalActs/CDE958CA-F272-4CB9-B8EA-2B85D6C3DB0A (Figures 3–5).

3.2.1. Studied Specimens. Holotype (male with pupal and larval exuviae) (reared) (Type number B.U. Ent. 269), India, West Bengal, Darjeeling (27°05′00″N; 88°26′67″E), 23/v/1996, N. Hazra leg; Paratypes (4 males with pupal exuviae) (reared), as holotype.

3.2.2. Etymology. From the Latin *spina*, spine, and *cornus*, horn, referring to the numerous/many spinules of the thoracic horn of the pupa.

3.2.3. Description

Adult Male (n = 5) (Figure 3). Total length 1.85–1.88 mm. Wing length 1.37–1.4 mm. Total length/wing length 1.34–1.35. Wing length/length of profemur 1.9–1.95. Thorax, abdomen and legs pale yellow.

Head. AR 0.31–0.38; flagellomere 13 (Fm 13) 106–110 μm long with a large seta 50–62 μm long. Eye with 42–50 μm long dorsomedial extension. Temporal setae 6-7, including 2 inner verticals (IV), 3 outer verticals (OV) and 1-2 postoculars (Po). Clypeus with 19–21 setae. Tentorium not measurable. Palpomere lengths (I–V): 18–20 μm; 26–34 μm; 56–68 μm; 86–102 μm; 98–126 μm.

Thorax. Acrostichals 12-13; dorsocentrals 11–13; scutellars 8.

Wing (Figure 3(a)). Membrane densely covered with setae, especially in distal half. Costal length 1.24 mm. Costa not extended. CR 0.90. VR 1.40. Sc, M and RM bare, *R* with 22–26 setae; R_1 28–32; R_{4+5} 64–66; M_{1+2} 48–52; M_{3+4} 104–108; Cu 32–34; Cu_1 22–26; PCu 46–48; An 25. Cell m with 5–10 setae, r_{4+5} about 200, m_{3+4} about 50, cu and an combined 25 setae. RM well proximal to FCu.

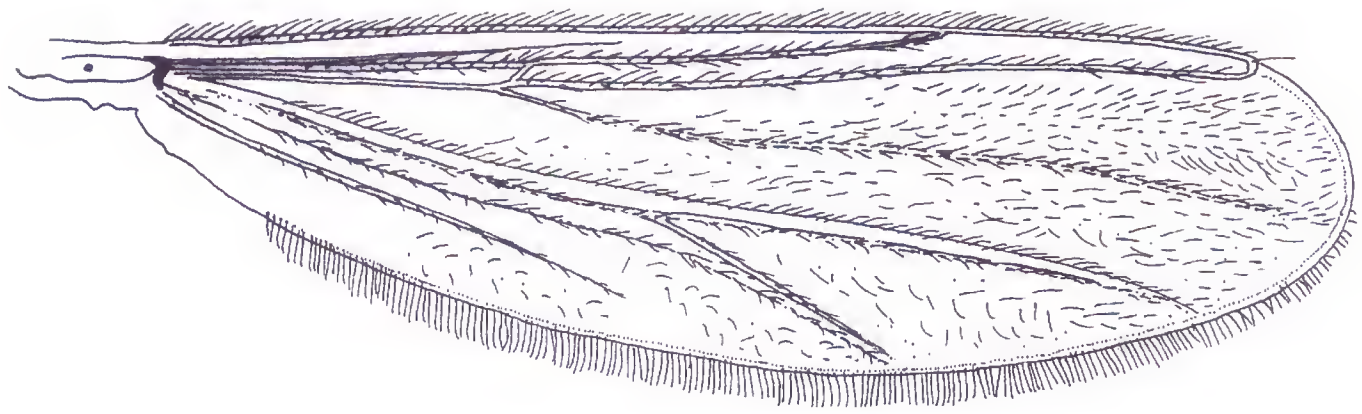
Legs. Spur of fore tibia (ti) 20–24 μm long; spurs of mid ti unequal 24–28 μm and 36–38 μm long including 12–14 of comb; spurs of hind ti 28–30 μm and 30–32 μm including 12–16 of comb. Width at the apex of fore tibia 26–28 μm; mid tibia 34–36 μm; hind tibia 32–36 μm. Lengths and proportions of leg segments as in Table 2.

Hypopygium (Figures 3(b)–3(f)). Tergite IX with 11–13 setae and prominent shoulder. Anal tergite band V-shaped with medially joined 33 μm long basal tergite band. Anal point spatulate 58–62 μm long, 8 μm wide at base, 6 μm wide at apex; crest nearly V-shaped, open. Phallapodeme 27 μm long, coxapodeme 45 μm long, lateral sternapodeme 57 μm long, transverse sternapodeme 54 μm long. Superior volsella (Figure 3(d)) 50–52 μm long, 15 μm wide, ovoid; median volsella (Figure 3(e)) 40–42 μm long with subulate setae fused into a plate with apical point not extending beyond both superior volsella and inferior volsella; inferior volsella (Figure 3(f)) 42–44 μm long with 7-8 setae and microtrichia. Gonocoxite 56–58 μm long; Gonostylus 84–88 μm long, abruptly narrowed distally. HR 0.66–0.67, HV 1.67.

Pupa (n = 5) (Figure 4). Total length 3.07–3.11 mm. Exuviae pale with outer edge of cephalothorax and margins of tergite VIII darker.

Cephalothorax. Frontal apotome (Figure 4(a)) rugulose. Frontal setae 75–80 μm long, seated medially, arising from tubercles. Thoracic horn (Figure 4(b)) 518–526 μm long, 35 width at base, without a median bend with many spinules in distal 2/3, horn arising from oval base. Thorax smooth, wing sheath with prominent nose (Figure 4(c)), 15–24 μm long. Two antepronotals, one median antepronotals 136–140 μm long and one lateral antepronotals 40–44 μm long. Three precorneals, anterior one 150–156 μm long, lamelliform; median one 60–66 μm long, lamelliform and posterior one 64–68 μm long. Dorsocentrals Dc_1 and Dc_2 paired 32–36 μm and 12–16 μm long respectively and Dc_3 and Dc_4 also paired, 62–70 μm and 14–18 μm long respectively; distance between two paired dorsocentrals 38–42 μm.

Abdomen (Figures 4(d)–4(g)). Tergite I bare. Tergites II–V with circular anterior pair of spines patches; posterior spinules on tergite II undivided (Figure 4(e)). Tergites III–V with shagreen next to circular patches extending over and beyond the first dorsal seta. Pair of circular patches on tergite V smaller than others. Median shagreen essentially



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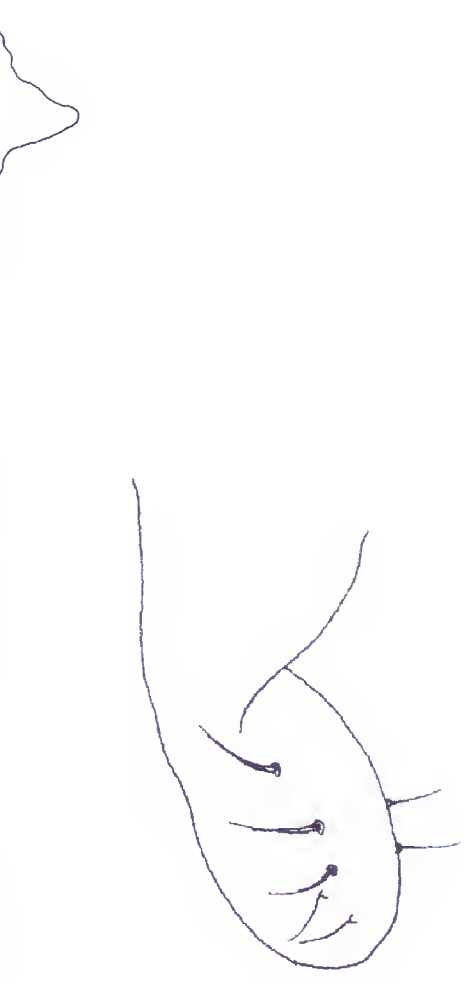

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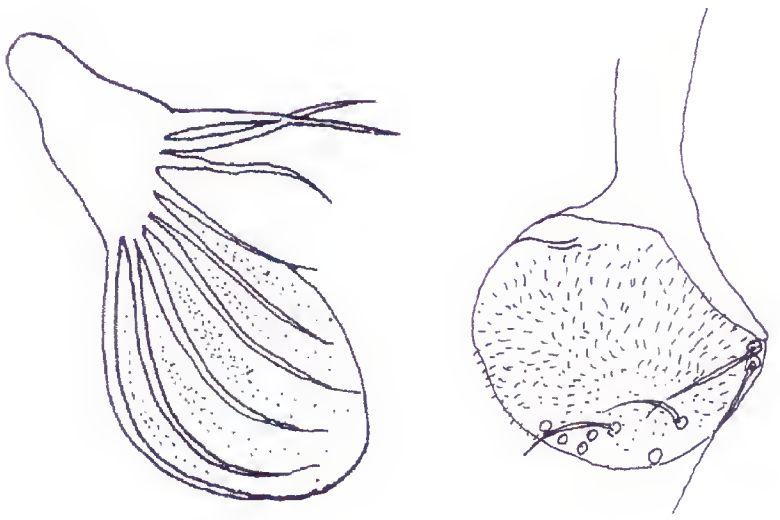


(e)

(f)

FIGURE 3: *Rheotanytarsus spinicornus* n. sp., adult male: (a) wing; (b) hypopygium (left-dorsal view); (c) hypopygium (right-ventral view); (d) superior volsella; (e) median volsella; (f) inferior volsella.

TABLE 2: Lengths (μm) and proportions of leg segments.

	fe	ti	ta_1	ta_2	ta_3	ta_4	ta_5	LR	BV	SV
P_1	676–690	360–374	870–884	420–434	286–300	240–254	106–120	2.36–2.42	1.76–1.82	1.19–1.20
P_2	660–674	510–524	270–286	136–150	106–120	60–74	46–60	0.53–0.54	3.67–4.14	4.19–4.34
P_3	690–704	616–630	450–464	226–254	210–224	136–150	60–74	0.73–0.74	2.56–2.78	2.87–2.90

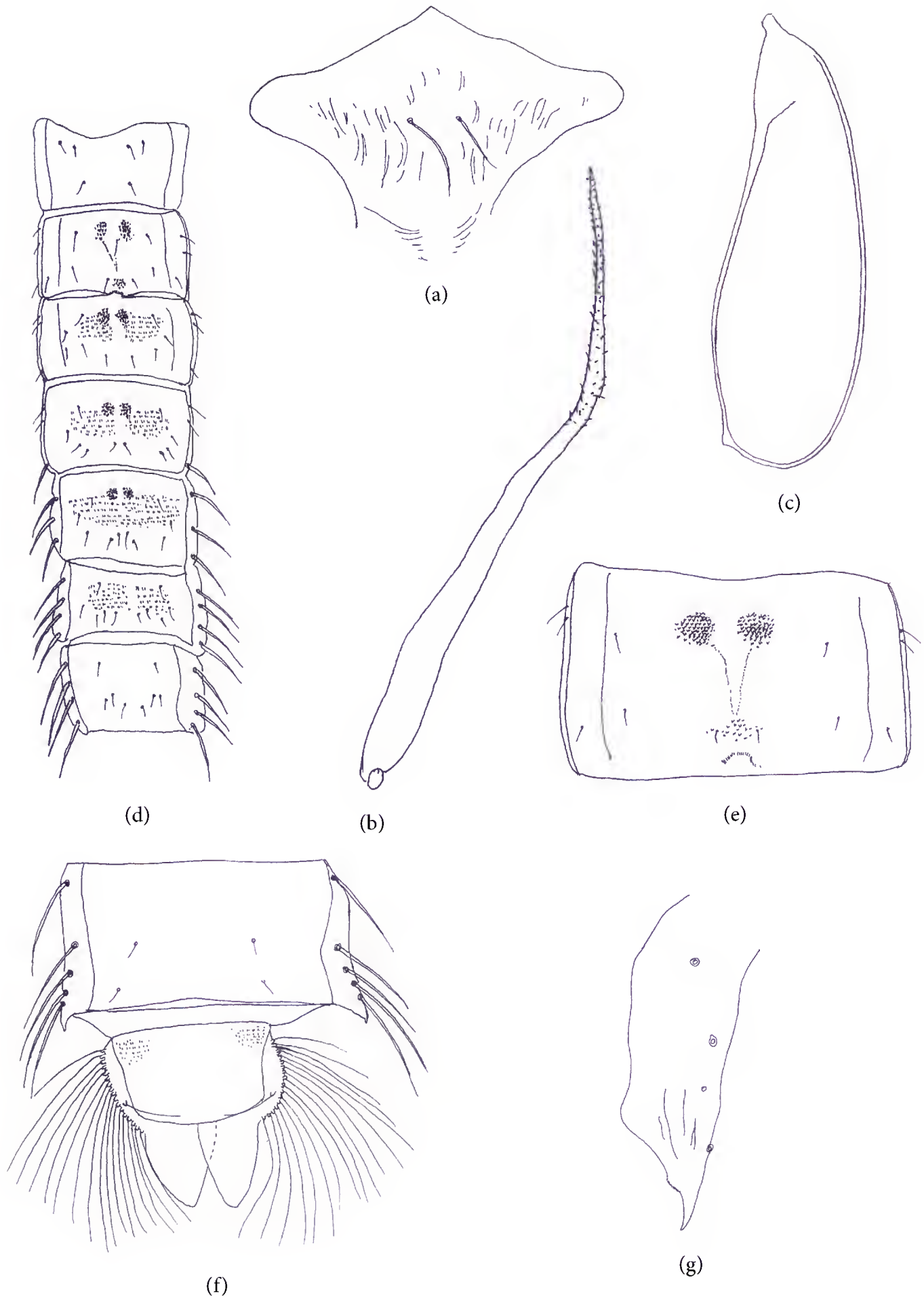


FIGURE 4: *Rheotanytarsus spinicornus* n. sp., pupa: (a) frontal apotome; (b) thoracic horn; (c) wing sheath; (d) tergites I–VII; (e) tergite II; (f) tergite VIII and anal lobe; and (g) caudolateral spur.

absent on tergites IV-V, weak and sparse shagreen present caudolaterally on tergites IV-V. Number of spines on patches on tergites II–V: 130–138; 100–110; 84–90; 50–54. Hook row small, not dividing medially, occupying 0.06 width of the segment width, containing about 26–30 hooklets. Conjunctives without shagreen. Segments II and III with 3 fine L setae; IV with 2 L setae and 1 posterior LS seta; V with 3 LS setae; VI-VII with 4 LS setae; VIII with 5 LS setae (Figure 4(f)). Caudolateral spur single (Figure 4(g)), 16–22 μm long. Shagreen present anterolaterally on anal lobe, 114–118 μm long and 180–186 μm wide with complete fringe of 34–36 lamelliform setae. Anal lobe (Figure 4(f)) with one dorsal seta, 32–36 μm long. ALR 1.26, G/F 1.71.

Larva (n = 2) (Figure 5). Total length 3.0–3.4 mm.

Antenna (Figure 5(a)). Length of antennal segments: 85–89 μm, 22–26 μm, 5–7 μm, 3.5–4.5 μm, 1.8–2.5 μm; AR 2.21–2.26; distance of ring organ and seta from base 1.85–2.96 μm and 5.55–6.29 μm respectively; style of segment II 3.7–5.5 μm long and opposite large Lauterborn organ 7.4–9.2 μm long placed on pedicels, not extending beyond antennal apex;

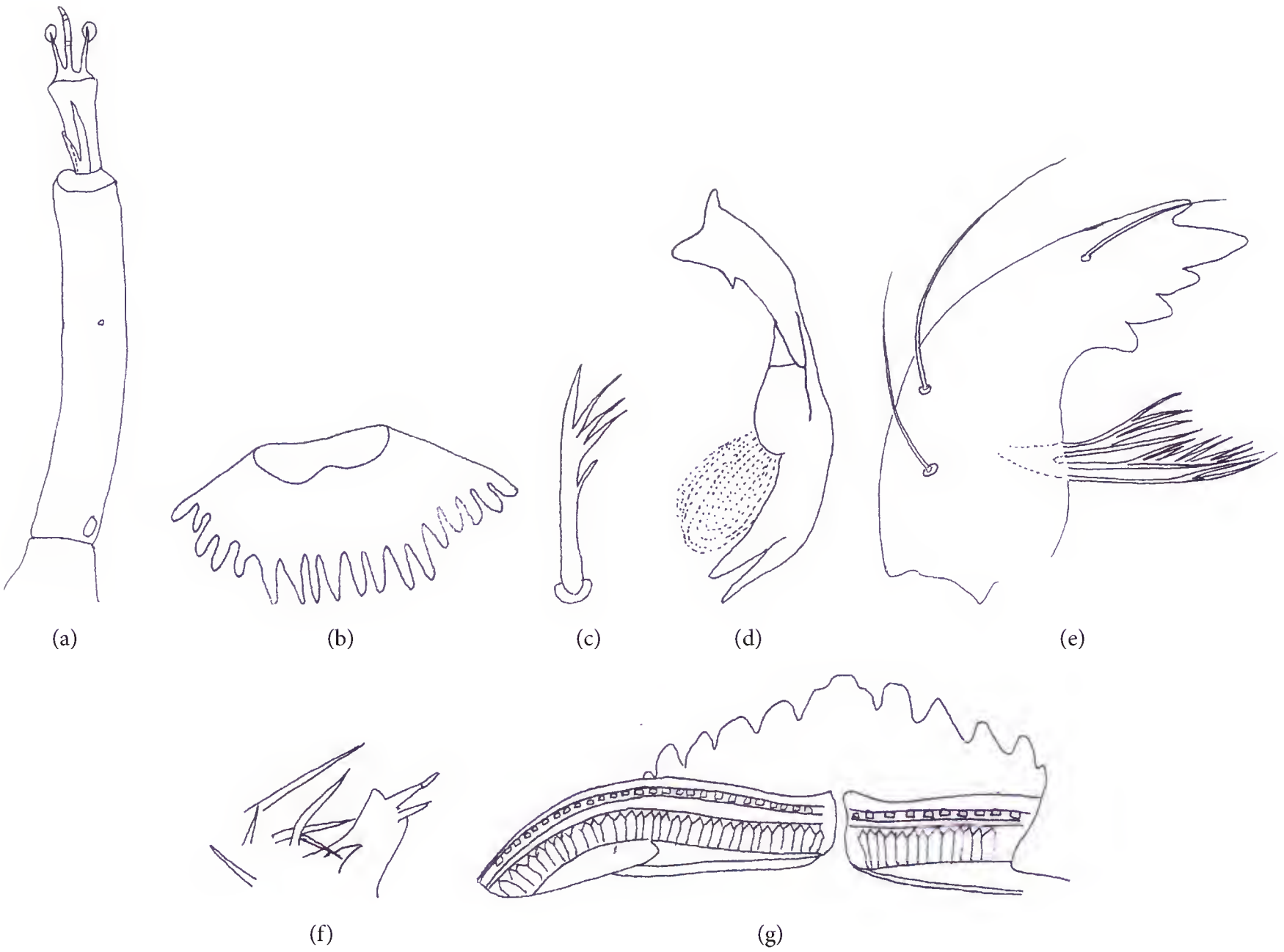


FIGURE 5: *Rheotanytarsus spinicornus* n. sp., larva: (a) antenna; (b) pecten epipharyngis; (c) S I of labrum; (d) premandible; (e) mandible; (f) maxillary palp; and (g) mentum.

blade 20–23 μm long with basally fused accessory blade 13–15 μm long.

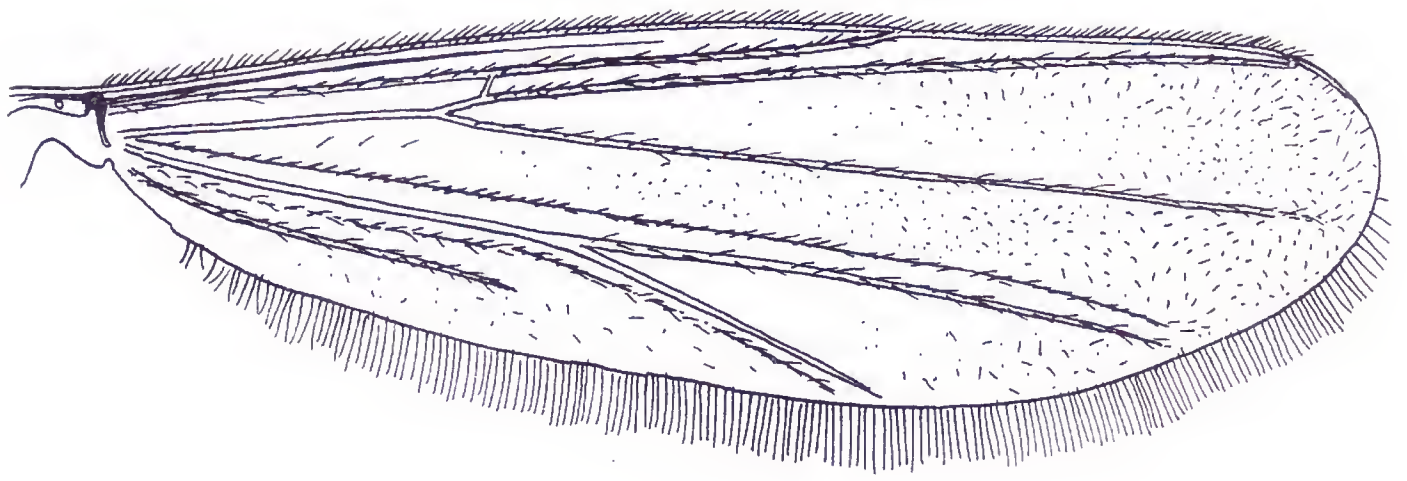
Labroepipharyngeal Region. Labral lamella 20–23 μm long and maximum width 24–29 μm; *pecten epipharyngis* (Figure 5(b)) a single broad distally serrated plate; S I as in the Figure 5(c); *premandible* (Figure 5(d)) 55–74 μm long, apically bifid. *Mandible* (Figure 5(e)) 92–96 μm long with 1 dorsal, 1 apical and 3 inner teeth; seta subdentalis 37–44 μm long reaching near the apex of mandible; seta interna with branches, largest branch 44–47 μm and shortest one 20–24 μm long.

Maxilla. Maxilla as in the Figure 5(f).

Mentum (Figure 5(g)). Median tooth 66–71 μm wide with 2 indistinct notches laterally; ventromental plate 64–67 μm long, separated by a narrow gap with rectangular 33–35 numbers of strial markings.

Body. Procercus 22–29 μm long with 7 setae. Anal tubules 103–111 μm long and 51–59 μm wide.

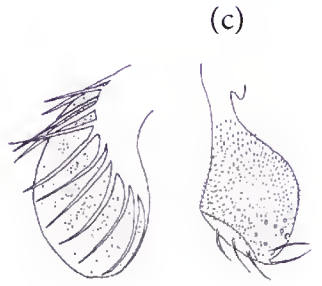
3.2.4. Remarks. *R. spinicornus* n. sp. belonging to the *muscicola* group is nearer to *R. foliatus* Kyerematen & Andersen [14] in basal tergite band but differs in shape of anal point. The pupa appears similar to those of *R. muscicola* Thienemann [15] and *R. photophilus* [16]. Larval characters such as antenna, mandible, premandible, and mentum show resemblances with *Rheotanytarsus* sp. 1 described by Roback & Coffman [17] from Nepal Alpine zone. In spite of the above similarities, the following combination of characters separates the new species from other members of the *muscicola* group in (i) V-shaped anal tergite band with medially joined basal tergite band, (ii) anal crest roughly V-shaped and open, (iii) median volsella not reaching the apex of superior volsella with subulate apical setae fused into a plate, (iv) gonostylus longer than gonocoxite and abruptly narrowed distally in adult male, (v) numerous spinules on distal 2/3 of thoracic horn without a median bend, (vi) tergites II–V with anterior pair of spines of circular patches, (vii) posterior spinules on tergite II undivided, (viii) anal lobe with hair-like dorsal seta on pupa, (ix) pecten epipharyngis, a single broad distally serrated plate, (x) median tooth of mentum with 2 indistinct notches laterally, and (xi) mandible with 3 inner teeth on larva.



(a)

(b) (c)

(d) (e)



(f) (g)

FIGURE 6: *Rheotanytarsus caputimberus* n. sp., adult male: (a) wing; (b) hypopygium (left-dorsal view); (c) hypopygium (right-ventral view); (d) shoulders at the posterior margin of tergite IX; (e) superior volsella; (f) median volsella; and (g) inferior volsella.

3.3. The trivittatus Group [6]

Emended Diagnosis. Median volsella reaching or not reaching beyond the apex of superior volsella and basal anal tergite band joined at the middle or interrupted in adult male.

Rheotanytarsus caputimberus n. sp. http://zoobank.org/NomenclaturalActs/044B8A86-BD12-4E4A-93D0-872184AA6201 (Figures 6-7).

3.3.1. Type Material. Holotype (male with damaged pupal exuviae) (reared) (Type No. B.U. Ent.270), India, Sikkim, Tadong (27°31′67″N; 88°60′00″E), 06/iv/1996, N. Hazra leg. Paratype (1 male), India, Sikkim, Ravangla (27°29′25″N; 88°35′94″E), 11/vii/2014, K. Sanyal leg.

3.3.2. Etymology. From the Latin *caput*, head and *imber*, shower, referring to shape of the inferior volsella similar to the

Table 3: Lengths (μm) and proportions of leg segments.

	fe	Ti	ta_1	ta_2	ta_3	ta_4	ta_5	LR	BV	SV
P_1	800	432	—	—	—	—	—	—	—	—
P_2	816	720	352	126	90	72	60	0.48	5.42	4.36
P_3	912	404	480	210	117	117	75	0.68	3.58	3.36

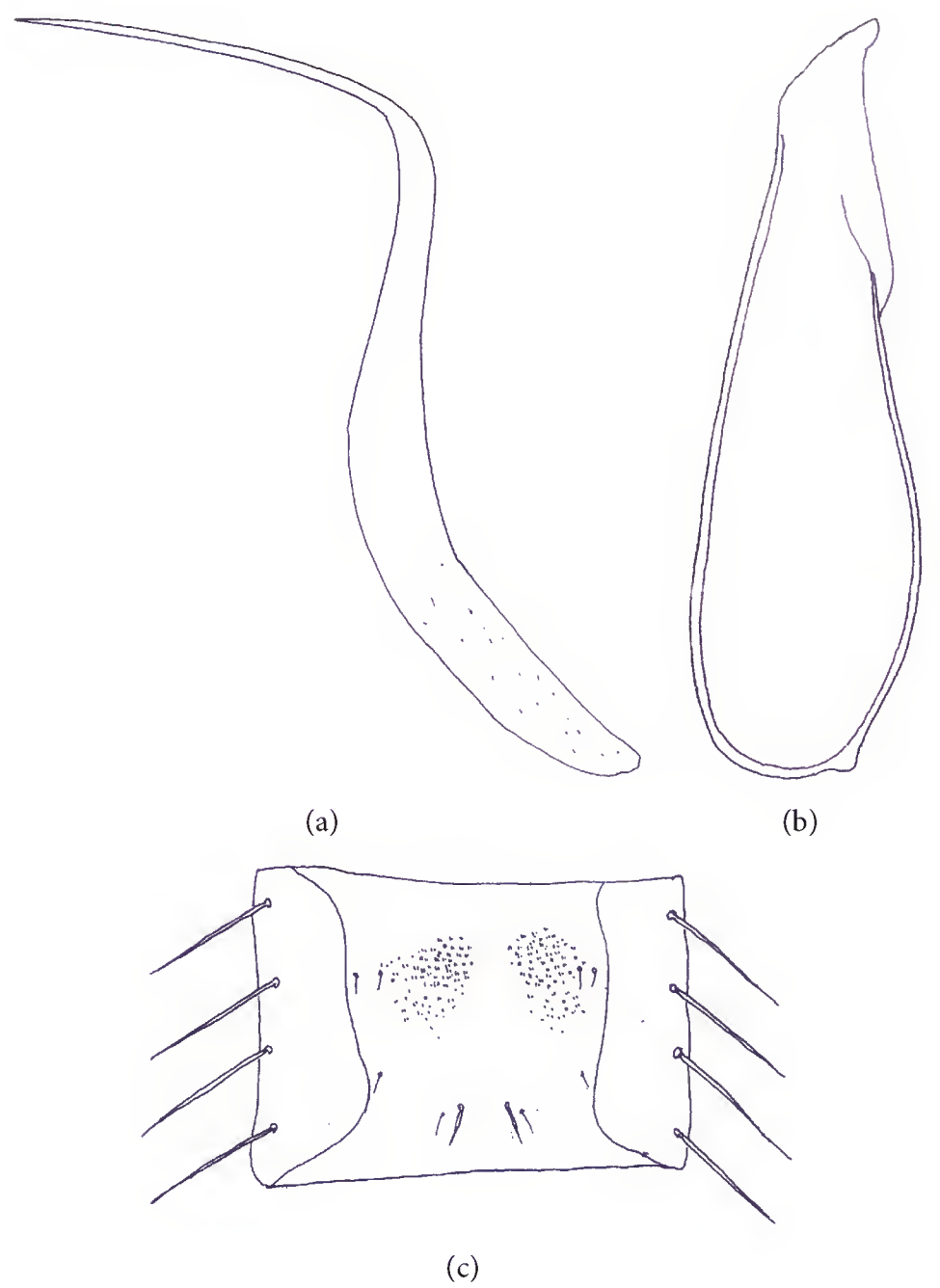


Figure 7: *Rheotanytarsus caputimberus* n. sp., pupa: (a) thoracic horn; (b) wing sheath; and (c) tergite VI.

head of hand shower, and the suffix – *us* denoting the gender of the genus.

3.3.3. Description

Adult Male (n = 2) (Figure 6). Total length 1.92–2.51 mm. Wing length 1.33–1.55 mm. Total length/wing length 1.44–1.61. Wing length/length of profemur 1.89–2.00. Thorax, abdomen and legs brownish yellow.

Head. AR 0.63–0.64; flagellomere 13 (Fm 13) 690 μm long. Eye with 90–96 μm long dorsomedial extension. Temporal setae 8, including 6 outer verticles (OV) and 2 postoculars (Po). Clypeus with 16–20 setae. Tentorium 66 μm long. Palpomere lengths (I–V): 21–24 μm; 27–30 μm; 72–75 μm; 72–84 μm; 144 μm.

Thorax. Acrostichals 12–14; dorsocentrals 8–11; scutellars 7-8.

Wing (Figure 6(a)). Membrane densely covered with setae, especially in distal half. Costal length 0.88–0.92 mm. Costa not extended. CR 0.67–0.92. VR 1.32–1.39. Sc, M and RM bare, *R* with 13–15 setae; R_1 20–23; R_{4+5} 44–52; M_{1+2} 30–33; M_{3+4} 22–28; Cu 13–18; Cu_1 16–21; PCu 45; An 25. Cell m with about 3 setae, r_{4+5} about 300, m_{3+4} about 34, cu and an combined 30 setae. RM well proximal to FCu.

Legs. Spur of fore tibia (ti) 21 μm long; spurs of mid tibia unequal 30 μm and 24 μm long including 17-18 of comb; spurs of hind ti 21 μm and 9 μm long including 14-15 of comb. Width at the apex of fore tibia 33 μm; mid tibia 36 μm; hind tibia 30 μm. Lengths and proportions of leg segments as in Table 3.

Hypopygium (Figures 6(b)–6(g)). Anal tergite band nearly V-shaped with medially joined 27–30 μm long basal tergite band. Tergite IX (Figure 6(d)) with projections to each side of the anal point, 3-4 setae on the dorsal side and 3 caudal setae including 2 setae in each projection. Anal point 36–45 μm long, 9–12 μm wide at base 3–6 μm wide at apex; crest not visible. Phallapodeme 30–36 μm long, transverse sternapodeme 18–20 μm long., lateral sternapodeme 36 μm long. Superior volsella (Figure 6(e)) 27–30 μm long, 6 μm wide at base, 15 μm wide at apex, ovoid; median volsella (Figure 6(f)) 10–12 μm long with subulate setae fused into a plate reaching at the apex of superior volsella; shape of the distal end of inferior volsella (Figure 6(g)) like the head of hand shower, 38–45 μm long with 12–14 setae, extending beyond the junction of gonocoxite and gonostylus. Gonocoxite 63–66 μm long; gonostylus 81–84 μm long, abruptly narrowed distally and parallel sided. HR 0.78, HV 2.6–2.98.

Pupa (n = 1) (Mostly Damaged) (Figure 7). Exuviae pale with outer edge of cephalothorax and margins of tergite VIII darker.

Cephalothorax. Frontal apotome rugulose. Frontal setae 35 long, seated medially, arising from tubercles. Thoracic horn (Figure 7(a)) 114 long, 7 width at base without any median bend, bearing few spinules at the basal region. Thorax smooth, wing sheath with prominent nose (Figure 7(b)), 21 long. Antepronotals two, median one 136–140 long and lateral

Table 4: Key to Adult Males of Oriental *Rheotanytarsus* Thienemann & Bause (Modified after Kyerematen et al. [2]).

(1) Antenna with 12 flagellomeres	2
Antenna with 13 flagellomeres	6
(2) Apical lamellae of median volsella bulbous, Oriental China	*bullus* Wang & Guo
Apical lamellae of median volsella not bulbous	3
(3) Apex of anal point broad, India, Malaysia	*madarihatensis* Kyerematen, Andersen & Sæther
Apex of anal point narrow	4
(4) Anal tergite bands nearly horizontal, Oriental China	*polychaetus* Wang & Guo
Anal tergite bands V-shaped	5
(5) Anal tergite with narrowly interrupted basal band, Malaysia	*kuantanensis* Kyerematen, Andersen & Sæther
Anal tergite without basal band, India	*nudicornus* n. sp.
(6) Median volsella long, reaching beyond apex of inferior volsella	7
Median volsella short, not reaching beyond apex of inferior volsella	10
(7) Median volsella apically with two distinct plates, Indonesia	*adjectus* (Johannsen)
Median volsella with distal lamellate apical setae never fused into plate	8
(8) Anal tergite band V-shaped, Thailand, Australia	*oss* Cranston
Anal tergite band transverse	9
(9) Anal tergite band separate; median volsella conspicuously slender, Thailand	*minusculus* Kyerematen, Andersen & Sæther
Anal tergite band medially fused; median volsella not slender, Oriental China, Ghana	*buculicaudus* Kyerematen in Kyerematen, Andersen & Sæther
(10) Posterior margin of tergite IX with projections or shoulders to each side	11
Posterior margin of tergite IX triangular, rounded, or at most straight	13
(11) Tergite IX posterior margin with well-developed projections; gonostylus abruptly tapered	12
Tergite IX posterior margin without such projections, but with distinct shoulders, gonostylus not abruptly tapered, Indonesia, Australia	*trivittatus* (Johannsen)
(12) Inferior volsella hand shower shaped; basal anal tergite band joined medially, India	*caputimberus* n. sp.
Inferior volsella not as above, basal anal tergite band interrupted, Indonesia	*additus* (Johannsen)
(13) Digitus well developed, extending beyond margin of superior volsella	14
Digitus small or absent, not extending beyond margin of superior volsella	21
(14) Median volsella not reaching apex of superior volsella; if gonostylus abruptly tapering distally, then apical portion parallel-sided and straight	15
Median volsella at least reaching apex of superior volsella; if gonostylus abruptly tapered, then apical portion not parallel-sided and straight	20
(15) Superior volsella knob- or hook-like posterior extension	16
Superior volsella rounded, ovoid, oblong or thumb-like	18
(16) Superior volsella with posterior extension knob-like, Thailand	*orientalis* Moubayed
Superior volsella with posterior extension hook-like	17
(17) Superior volsella greatly hooked; apical setae of superior volsella not fused into plate; Thailand, Lebanon, Europe	*reissi* Lehmann
Superior volsella slightly hooked; apical setae of superior volsella fused into plate; Oriental and Palaearctic China, Europe, North Africa and Canada	*muscicola* Thienemann
(18) Anal point tapering; Oriental Japan	*amamiflavus* Sasa
Anal point spatulate	19
(19) Superior volsella rectangular with rounded margin; basal tergite band medially joined; Oriental China, Palaearctic Japan	*tamatertius* Sasa
Superior volsella rounded; basal tergite band absent; Oriental Japan	*okisimplex* Sasa

Table 4: Continued.

(20) Gonostylus abruptly tapered with apical portion curved; superior volsella with large, bluntly rounded apical projection; median volsella apparently without apical plate, India, Indonesia, Oriental China	*acerbus* Johannsen
Gonostylus not abruptly tapered; superior volsella subquandrangular; median volsella with wide apical plate; Palaearctic Japan, Oriental and Palaearctic China	*tamaquartus* Sasa
(21) Abdomen banded; anal point crests long, proximally fused forming an arc; superior volsella rounded; median volsella without plate	22
Abdomen not banded; hypopygium not with above configuration	23
(22) Gonostylus not abruptly tapered plate, India, Indonesia, Oriental and Palaearctic Japan, Palaearctic China	*aestuarius* (Tokunaga)
Gonostylus abruptly tapered, Oriental China	*liuae* Wang & Guo
(23) Gonostylus more or less abruptly tapered, with distinctly parallel-sided apical portion or with curved apex; superior volsella with pronounced posterior extension; median volsella extending beyond apex of superior volsella	24
If gonostylus abruptly tapered, then without parallel-sided or curved apex	25
(24) Gonostylus with apical portion distinctly parallel-sided; superior volsella with posterior extension long, digitiform; Malaysia	*phaselus* Kyerematen, Andersen & Sæther
Gonostylus with apex curved; superior volsella with posterior extension broad and rounded; Thailand	*thailandensis* Moubayed
(25) Anal tergite bands V-shaped and medially joined	26
If anal tergite bands V-shaped, then not medially joined	27
(26) Anal point nonspatulate; median volsella extremely beyond apex of superior volsella; AR > 0.60, Indonesia	*tobaseptidecimus* Kikuchi & Sasa
Anal point spatulate; median volsella not extending beyond apex of superior volsella; AR < 0.40, India	*spinicornus* n. sp.
(27) Apex of anal point spatulate; gonostylus tapering abruptly or gradually	28
Apex of anal point parallel-sided or tapering; gonostylus tapering gradually	34
(28) Superior volsella with pronounced, hook-like posterior extension, Thailand	*falcipedius* Kyerematen, Andersen & Sæther
Superior volsella rounded or rectangular	29
(29) Superior volsella rectangular, Oriental China	*quadratus* Wang & Guo
Superior volsella rounded	30
(30) Gonostylus tapering gradually	31
Gonostylus abruptly tapered in apical portion	33
(31) Anal point crests proximally fused, forming an arc, Thailand	*beccus* Kyerematen, Andersen & Sæther
Anal point crest V-shaped	32
(32) Median volsella reaching beyond apex of superior volsella; basal anal tergite bands absent; Thailand, Lebanon, Europe	*curtistylus* (Goetghebuer)
Median volsella short, not reaching apex of superior volsella; basal anal tergite bands present, Thailand	*falcatus* Kyerematen, Andersen & Sæther
(33) Median volsella recurved; AR about 0.70, Thailand	*sessilipersonatus* Kyerematen, Andersen & Sæther
Median volsella not markedly recurved; AR about 0.40, Thailand	*soelii* Kyerematen, Andersen & Sæther
(34) Median volsella extending beyond apex of superior volsella; anal point crests proximally fused, forming an arc, Thailand	*pallidus* Kyerematen, Andersen & Sæther
Median volsella not reaching beyond apex of superior volsella; anal point crests V-shaped	35
(35) Anal point broad, parallel–sided; AR about 0.1–0.3, Thailand	*koraensis* Kyerematen, Andersen & Sæther
Anal point lanceolate; AR about 0.3–0.4, Thailand	*verticillus* Kyerematen, Andersen & Sæther

Table 5: Key to Pupae of Oriental *Rheotanytarsus* Thienemann & Bause (Modified after Kyerematen et al. [2]).

(1) Tergite VIII with caudolateral comb; T II–V with anterior paired patches of spinules; thoracic horn with knee-like bend, heavily sclerotized	2
Tergite VIII with single spur; T II–IV, II–V or II–VI with paired patches of spinules; thoracic horn may be sharply bent but not knee-like	3
(2) Hook row of about 90 hooklets; anal lobe fringe of much less than 20 taeniae	*additus* (Johannsen)
Hook row of 60–70 hooklets; anal lobe fringe of about 20 taeniae	*trivittatus* (Johannsen)
(3) Thoracic horn sharply bent at midlength; T II–IV with oral, paired, rounded patches of spinules	4
Thoracic horn not sharply bent; T II–V or II–VI with spinule patches	5
(4) Tergite VIII with 3 lateral taeniae	*thailandensis* Moubayed
Tergite VIII with 5 lateral taeniae	*oss* Cranston
(5) Tergites II–VI with sharply defined, paired spinule patches	6
Tergites II–V with sharply defined patches of spinules	7
(6) Anal lobe with one large dorsal seta; thoracic horn with few fine spinules	*curtistylus* (Goetghebuer)
Anal lobe with two short dorsal setae; thoracic horn with many fine spinules in distal half	*orientalis* Moubayed
(7) Anal lobe without dorsal setae; distal half of thoracic horn with many spinules	*adjectus* (Johannsen)
Anal lobe with long dorsal setae; distal half of thoracic horn with or without any spinules	8
(8) Distal half of thoracic horn without any spinules	*nudicornus* n. sp.
Distal half of thoracic horn with few to many spinules	9
(9) Tergites II and III with transversely elongated or rectangular spine patches	*reissi* Lehmann
Tergites II-III with circular or elliptical spine patches	10
(10) Numerous spinules on distal 2/3 of the thoracic horn	*spinicornus* n. sp.
Spinules few on distal 1/3 of the thoracic horn	*tamaquartus* Sasa

one 40–44 long. Precorneals three, anterior one 72 long, lemelliform; median one 48 long, lamelliform and posterior not seen. Dorsocentrals Dc_1 and Dc_2 paired 12 and 9 long respectively and Dc_3 and Dc_4 also paired, 12 and 18 long respectively; distance between two paired dorsocentrals 51.

Abdomen. Tergites II–V with circular anterior pair of spine patches. Tergites III–V with shagreen next to circular patches extending over and beyond the first dorsal seta. Pair of circular patches on tergite V smaller than others. Number of spines on patches on tergites II–V: 75–80; 67–70; 38–40; 34-35. Hook row small, occupying 0.057 width of the segment, containing about 12-13 hooklets. Most of the abdominal segments including anal lobe damaged except segment VI (Figure 7(c)); segment V with 4 LS setae; VI 4 LS setae.

3.3.4. Remarks. Rheotanytarsus caputimberus n. sp. shows affinity with other members of the group having projections to each side of the anal point except *R. scutulatus* Kyerematen & Andersen [14]. Similarly superior volsella of the new species is similar to other members of the group except *R. ramirezae* Kyerematen [14] and *R. scutulatus* Kyerematen & Andersen [14]. Gonostylus is abruptly tapered like other members except in *R. ceratophylli* [18] and *R. trivittatus* Johannsen [19] In spite of the above, the proposed species may be separated from other members of the *trivittatus* group including oriental species *R. brevipalpus* Wang & Guo [7], *R. additus* [19], and *R. trivittatus* Johannsen and may be diagnosed by the following combination of characters: (i) lateral projections on each side of the anal point, (ii) basal anal tergite band medially joined, (iii) superior volsella ovoid, (iv) median volsella not reaching beyond apex of superior volsella with subulate setae fused into plate, (v) inferior volsella typically like head of hand shower, and (vi) gonostylus abruptly tapered.

Key to adult males of Oriental *Rheotanytarsus* Thienemann and Bause (modified after Kyerematen et al. [2]) is shown in Table 4 while Table 5 shows key to pupae of Oriental *Rheotanytarsus* Thienemann and Bause (modified after Kyerematen et al. [2]).

Disclosure

The new names included in this paper are available under the International Code of Zoological Nomenclature. This work and the nomenclatural acts it contains have been registered in ZooBank. ZooBank Life Science Identifier (LSID) for this publication is http://zoobank.org/References/9D3AF92A-43A6-4EE1-9319-3C4216740C12. The LSID registration and any associated information can be viewed in a web browser by adding the LSID to the portal "http://zoobank.org/."

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Functional Responses of *Nephus arcuatus* Kapur (Coleoptera: Coccinellidae), the Most Important Predator of Spherical Mealybug *Nipaecoccus viridis* (Newstead)

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Nephus arcuatus Kapur is an important predator of *Nipaecoccus viridis* (Newstead), in citrus orchards of southwestern Iran. This study examined the feeding efficiency of all stages of *N. arcuatus* at different densities of *N. viridis* eggs by estimating their functional responses. First and 2nd instar larvae as well as adult males exhibited a type II functional response. Attack rate and handling time were estimated to be 0.2749 h^{-1} and 5.4252 h, respectively, for 1st instars, 0.5142 h^{-1} and 1.1995 h for 2nd instars, and 0.4726 h^{-1} and 0.7765 h for adult males. In contrast, 3rd and 4th instar larvae and adult females of *N. arcuatus* exhibited a type III functional response. Constant *b* and handling time were estimated to be 0.0142 and 0.4064 h for 3rd instars, respectively, 0.00660 and 0.1492 h for 4th instars, and 0.00859 and 0.2850 h for adult females. The functional response of these six developmental stages differed in handling time. Based on maximum predation rate, 4th instar larvae were the most predatory (160.9 eggs/d) followed by adult females (84.2 eggs/d). These findings suggest that *N. arcuatus* is a promising biocontrol agent of *N. viridis* eggs especially for 4th instar larvae and adult females.

1. Introduction

The spherical mealybug, *Nipaecoccus viridis* (Newstead) (Hemiptera: Pseudococcidae), is one of the most important citrus pests in southern and southwestern Iran [1]. This polyphagous pest attacks over 193 plant species throughout tropical and subtropical regions and a large part of the Pacific Basin [2–4]. Chemical control of *N. viridis*, as with other mealybugs, often becomes ineffective due to their cryptic life style in protected locations as well as the presence of a mealy wax that covers its eggs and body [5]. Therefore, biological control using natural enemies has the potential to be an effective alternative method to manage this pest [6–8].

The coccidophagous coccinellid, *Nephus arcuatus* Kapur (Coleoptera: Coccinellidae), is a newly recorded predatory beetle indigenous to the warmer regions of Iran [9]. Until recently, it had only been reported in Yemen and Saudi Arabia [10]. This small coccinellid occurs widely and abundantly in citrus orchards in Dezful, southwestern Iran (personal observation). Recent investigations on the biology and consumption capacity of this predator confirm its potential for the control of *N. viridis* in the citrus orchards [11, 12]. However, more studies are needed to develop this predator within a successful biological management programme.

Prior to using a natural enemy in a biological control programme, it is essential to evaluate its predatory capacity. One of the criteria for determining the efficiency of a predator is the ability of the predator to change its feeding behaviour in response to changes in prey density, that is, its functional response, defined as the number of prey eaten per predator as a function of prey density [13, 14]. Several types of functional response curve have been described, including a linear increase (type I), an increase decelerating to a plateau (type II), or a sigmoidal increase (type III) in which predators cause a constant (I), negative (II), or positive (III) density-dependent mortality of their prey [13, 15, 16].

The functional response curve can be described by evaluating two parameters, the coefficient of attack rate (a) and the handling time (T_h). The coefficient of attack rate estimates the steepness of the increase in predation with increasing prey density and the handling time helps estimate the satiation threshold [16]. Information on these variables can provide insights into the efficiency of a predator in regulating prey populations, clarifying evolutionary relationships, and predicting the predator's effectiveness as a biological control agent [16–18].

This study aimed to determine the relative efficiency of different larval instars and of both female and male adults of *N. arcuatus* as biological control agents of *N. viridis*. We achieved this by evaluating the effect of *N. viridis* density on the number of prey consumed by each life stage of *N. arcuatus* to determine the shape of their functional response to prey density, their attack rate coefficients, and handling times.

2. Materials and Methods

2.1. Prey and Predator Cultures. *N. viridis* mealybugs were collected from *Citrus sinensis* L. trees in an orchard in Dezful (48°30′E, 32°20′N), Khuzestan Province, southwestern Iran, in the autumn of 2011. They were then mass-reared on sprouting potato (*Solanum tuberosum* L.) shoots, in rearing boxes (24 × 16 × 10 cm) that were tightly covered by a fine mesh net. *N. arcuatus* adults were collected from the same orchard and reared on sprouted potatoes infested with *N. viridis* for two generations before being used in experiments. The stock colonies of both *N. arcuatus* and *N. viridis* were maintained in an incubator at 30 ± 1°C, 65 ± 5% RH, and 14L : 10D photoperiod.

2.2. Functional Response Assessments. To obtain a cohort of *N. arcuatus* for experiments, 50 pairs of adult *N. arcuatus* were transferred from the stock culture into a colony of *N. viridis* (mixed developmental stages on 10–12 sprouted potato plants) in a plastic box (20 × 13 × 8 cm) covered with a fine mesh net for ventilation; predator oviposition was allowed to proceed for 12 h after which time the adult predators were removed. Developing predator larvae were observed every 12 h and, over time, developed into cohorts of 1st, 2nd, 3rd, and 4th instar larvae and mated adults males and females (10-day-olds) for use in experiments. Before each developmental stage was evaluated, replicate individuals were kept without food for 12 h in a micro tube (1.5 mL) in order to standardize their hunger level. Thereafter, each predator was introduced into a plastic container (9 × 7 × 3 cm) containing different densities of eggs of *N. viridis* which were the preferred prey for the developmental stage of *N. arcuatus* [19]. Each container had a 20 mm diameter hole in the middle of the lid, which was covered by a piece of fine net to provide ventilation. The densities of *N. viridis* eggs were as follows: 2, 4, 6, 8, 10, 14, and 18 eggs for 1st instar larvae; 2, 4, 8, 16, 20, 30, 40, and 50 eggs for 2nd instar larvae; 2, 4, 8, 16, 20, 40, 60, 80, 100, and 120 eggs for 3rd instar larvae; 2, 4, 8, 16, 32, 60, 100, 140, 180, and 220 eggs for 4th instar larvae; 2, 4, 8, 16, 40, 65, 90, and 115 eggs for adult females; and 2, 4, 8, 16, 20, 35, 50, 60, and 80 eggs for adult males. These densities were selected based on preliminary tests of the consumption capacity of different stages of *N. arcuatus*. After 24 h, predators were removed and the number of eggs consumed was recorded. There were between 10 and 21 replicates for each treatment; greater replication was used for some prey densities to achieve precise information. Experimental conditions were based on optimal temperature for *N. arcuatus* activity: 30 ± 1°C, 65 ± 5% RH, and 14L : 10D photoperiod [11].

2.3. Statistical Analysis. The functional responses of *N. arcuatus* were analyzed in two steps [20]. In the first step, the type (shape) of functional response was described by determining how well the data fitted to a type I, II, or III functional response, using a polynomial logistic regression of the proportion of prey consumed (N_a/N_0) as follows:

$$\frac{N_a}{N_0} = \frac{\exp\left(P_0 + P_1 N_0 + P_2 N_0^2 + P_3 N_0^3\right)}{1 + \exp\left(P_0 + P_1 N_0 + P_2 N_0^2 + P_3 N_0^3\right)}, \quad (1)$$

where N_a is the number of prey consumed, N_0 is the initial prey density, and the parameters P_0, P_1, P_2, and P_3 are the constant, linear, quadratic, and cubic parameters related to the slope of the curve. The above parameters were estimated using the CATMOD procedure in SAS software [20, 21]. The data sets for each developmental stage of *N. arcuatus* were fitted individually to (1) and the types of functional response were determined by examining the signs of P_1 and P_2. If P_1 was positive and P_2 was negative, a type III functional response was evident. However, if P_1 was negative the functional response type was a type II [20].

In the second step, a nonlinear least squares regression (PROC NLIN [21]) was used to estimate the functional response parameters (T_h and either a for type II functional response or b, c, and d for type III functional response) using Rogers's random predator equation which is the most appropriate type II functional response in situations with prey depletion [22]:

$$N_a = N_0 \left\{1 - \exp\left[a\left(T_h N_0 - T\right)\right]\right\}, \quad (2)$$

where T is the total time that predator and prey are exposed to each other (24 h); a is the attack rate; and T_h is the handling time in hours [20, 23].

For modeling the type III functional response, attack rate (a) in (2) was substituted in (3) with a function of prey density [16, 24]. In the simplest generalized form, attack rate (3) is a function of the initial number of prey:

$$a = \frac{\left(d + bN_0\right)}{\left(1 + cN_0\right)}, \quad (3)$$

where b, c, and d are constants that must be estimated. The simplest form arises when a is a function of initial density, as in

$$N_a = N_0 \left\{1 - \exp\left[\left(d + bN_0\right)\left(T_h N_a - T\right)\left(1 - cN_0\right)\right]\right\}. \quad (4)$$

The functional response parameters for 1st instar and 2nd instar larvae and adult males were obtained using (2) (for type II). However, the functional response parameters for 3rd instar and 4th instar larvae and adult females were obtained using (3) and (4) (for type III).

Differences in estimates of attack rates and handling were analyzed using (5) (type II) or (6) (type III) with an indicator variable as follows [20]:

$$N_a = \left\{1 - \exp\left[-\left(a + D_a(j)\right)\left(T - \left(T_h + D_{T_h}(j)\right)N_a\right)\right]\right\}, \quad (5)$$

$$N_a = \frac{\exp\left[b + D_b(j)\right]N_0^2 T}{1 + \exp\left[b + D_b(j)\right]N_0^2\left[T_h + D_{T_h}(j)\right]}, \quad (6)$$

where j is an indicator variable that takes the value 0 for the first data sets and the value 1 for the second data sets. For a type II response (5), the parameters D_a and D_{T_h} estimate the differences between the data sets in the values of the parameters a and T_h, respectively. Specifically, the attack rate for one stage is a, and that for another stage is $a + D_a$. If the parameters D_a and D_{T_h} are significantly different from zero, then a and T_h, for the two data sets, are different. For the type III response (6), the parameters D_b and D_{T_h} estimate the differences between the two data sets being compared with the values of b and T_h, respectively. Specifically, the handling time for one stage is T_h, and that for another stage is $T_h + D_{T_h}$ [20].

The maximum predation rate (T/T_h), which represents the maximum number of prey that can be consumed by an individual during 24 h, was calculated using the estimated T_h [25].

3. Results

The polynomial logistic regression analysis of the proportion of *N. viridis* consumed by 1st and 2nd instar larvae and by adult male *N. arcuatus* yielded estimated parameters that indicate a type II functional response for these predator stages (Table 1). The linear coefficient P_1 was negative for these stages; that is, the proportion of prey consumed declined monotonically with an increase in the initial number of prey offered, which indicates a type II functional response (Figures 1 and 2). Therefore, (2) was used to estimate a and T_h. Estimated parameters showed that the 1st instar larva of *N. arcuatus* had the smallest attack rate and handling time compared with 2nd instar larva and adult males (Table 2). The asymptotic 95% confidence interval for D_a included 0 but that for D_{T_h} did not, which means that there is a significant difference between T_h and $T_h + D_{T_h}$ and that these three groups have a different functional response, with a significant difference in handling time, but not in attack rate (Table 3).

Results of the polynomial logistic regression for 3rd and 4th instar larvae and adult female *N. arcuatus* indicate a type III functional response for these predator stages. The linear coefficient, P_1, was positive, and the quadratic coefficient, P_2, was negative for these stages (Table 1). Thus, the proportion of prey consumed is positively density dependent, which indicates a type III functional response (Figures 1 and 2). Therefore, (3) was substituted for (2), and the two data sets were fitted to a type III functional response curve. Results of the nonlinear least square regression indicated that parameters c and d were not significantly different from 0 (not shown): therefore, they were removed from the model, and a reduced model was used [20]. Similarity relationships between a and N_0 in these data sets ($a = bN_0$) enabled the use of model (6) for data analysis. Both the estimated b value and T_h were smallest for 4th instar larvae followed by adult females. The asymptotic 95% confidence interval for D_b and D_{T_h} showed that there was a significant difference between T_h and $T_h + D_{T_h}$, while there is no significant difference between b and $b + D_b$ (Table 4). Thus, there is a significant difference between T_h and $T_h + D_{T_h}$ and these three groups also have a different functional response, with a significant difference in handling time, but not in constant b (Table 3). For these three groups the relationships between the attack rate and the initial number of prey were linear ($a = bN_0$), and the rate of successful attack (a) ranged from 0.0284 to 1.704 h^{-1} for 3rd instar larvae; from 0.0132 to 1.452 h^{-1} for 4th instar larvae; and from 0.0172 to 0.6872 h^{-1} for adult females.

The value of the coefficient of determination (R^2 = 1 – residual sum of squares/corrected total sum of squares) indicated that Rogers's random predator equations ((2) and (4)) adequately described the functional responses of all stages of *N. arcuatus* (see values for R^2, Table 2).

The maximum number of *N. viridis* eggs that could be eaten by all stages of *N. arcuatus* increased with larval instar and adult females consumed more *N. viridis* eggs than adult males (Table 2). This parameter was highest for 4th instar larva followed by adult females and 3rd instar larva.

4. Discussion

The warm, dry climate of southwestern Iran provides suitable conditions for activity of the mealybug, *N. viridis*, in orchards [1]. In these regions, as in many countries, *Cryptolaemus montrouzieri* Mulsant is released to control mealybugs in orchards. However, probably due to warm summers and the symbiotic relationship between ants and mealybugs, this predator has not been able to establish permanent populations and must be mass-reared and released on a yearly basis to control *N. viridis* [8]. In contrast, *N. arcuatus* is the most abundant predator in orchards from spring to fall and often controls *N. viridis* in citrus orchards (personal observation). Zarghami et al. [11] studied the effect of temperature on the population growth and life table parameters of *N. arcuatus* as a predator of *N. viridis* and noted that *N. arcuatus* could develop at a wide range of temperatures (20–35°C), with an optimal temperature of 30°C. They reported that when *N. arcuatus* was provided with two prey species (*N. viridis* and *P. citri*), prey stage, prey size, and previous feeding experience had no effect on prey selection by this predator [12]. Moreover, *N. arcuatus* is considered to be the most effective predator of other mealybugs including *Maconellicoccus hirsutus* (Green) and *Phenacoccus solenopsis* Tinsley due to its large populations and extended periods of activity, especially during the hot summer months [26, 27].

Table 1: Results of logistic regression analysis of the proportion of *N. viridis* consumed by different stages of *N. arcuatus* as a function of initial prey density.

Stages of predator	Coefficient	Estimate	SE	χ^2	P value
1st instar	Constant	3.4233	1.0793	10.06	0.0015
	Linear	−0.5926	0.3537	2.81	0.0939
	Quadratic	0.0273	0.0351	0.61	0.4359
	Cubic	−0.00048	0.00106	0.21	0.6474
2nd instar	Constant	9.6343	1.9010	25.69	<0.0001
	Linear	−0.6126	0.1849	10.98	0.0009
	Quadratic	0.0131	0.00565	5.39	0.0202
	Cubic	−0.00010	0.000055	3.23	0.0724
3rd instar	Constant	3.1992	0.3966	65.07	<0.0001
	Linear	0.0411	0.0193	4.56	0.0326
	Quadratic	−0.00143	0.000286	24.96	<0.0001
	Cubic	$7.195E-6$	$1.281E-6$	31.55	<0.0001
4th instar	Constant	4.0195	0.6154	42.67	<0.0001
	Linear	0.0686	0.0162	18.04	<0.0001
	Quadratic	−0.00080	0.000129	38.68	<0.0001
	Cubic	$1.927E-6$	$3.044E-7$	40.09	<0.0001
Adult females	Constant	3.1257	0.6513	23.03	<0.0001
	Linear	0.1424	0.0358	15.78	<0.0001
	Quadratic	−0.00311	0.000575	29.35	<0.0001
	Cubic	0.000015	$2.681E-6$	30.64	<0.0001
Adult males	Constant	13.4559	1.9436	47.93	<0.0001
	Linear	−0.6228	0.1122	30.79	<0.0001
	Quadratic	0.00970	0.00206	22.07	<0.0001
	Cubic	−0.00005	0.000012	17.88	<0.0001

Table 2: Parameters estimated by Rogers's random predator equation as well as R^2 and maximum predation rate (T/T_h) for six life stages of *N. arcuatus* fed on *N. viridis*.

Stages of predator	Parameter	Estimate	Approximate SE	Approximate 95% confidence		T/T_h	R^2
				Lower	Upper		
1st instar	a	0.2749	0.1132	0.0493	0.5004	4.4	0.999
	T_h	5.4252	0.2844	4.8583	5.9921		
2nd instar	a	0.5142	0.1662	0.1841	0.8443	20	0.982
	T_h	1.1995	0.0375	1.1249	1.2740		
3rd instar	b	0.0142	0.00344	0.00739	0.0210	59.2	0.989
	T_h	0.4064	0.00604	0.3945	0.4184		
4th instar	b	0.00660	0.00244	0.00177	0.0114	160.9	0.986
	T_h	0.1492	0.00403	0.1412	0.1572		
Adult females	b	0.00859	0.00265	0.00333	0.0138	84.2	0.978
	T_h	0.2850	0.00978	0.2656	0.3044		
Adult males	a	0.4726	0.2252	0.0254	0.9199	30.9	0.976
	T_h	0.7765	0.0318	0.7133	0.8398		

The current study is the first to assess the efficiency of all stages of *N. arcuatus* as a predator of *N. viridis*. We found that different developmental stages of *N. arcuatus* had different types of functional responses. The 1st and 2nd instar larvae as well as adult males exhibited a type II functional response. In contrast, the 3rd and 4th instar larvae and adult females exhibited a type III functional response. This is in accordance with observations for other insects species where the type of functional response and its parameters are affected by developmental stage [28–30]. For example, Bayoumy [28] found that the functional responses of 2nd instar larvae (type II), 4th instar larvae (type III), and adult females (type III) of *Nephus includens* (Kirsch) to *Aphis gossypii* Glover were markedly different. However, Tang et al. [31] and Milonas

Table 3: Parameters estimated by an equation with an indicator variable for comparing type II functional response parameters of 1st and 2nd instar larvae and males of *N. arcuatus*.

Comparison stage	Estimate	Approximate SE	Approximate 95% confidence	
			Lower	Upper
1st instar-2nd instar				
D_a	0.2393	0.2465	−0.2473	0.7260
D_{T_h}	**−4.2258***	**0.5222**	**−5.2567**	**−3.1948**
1st instar-male adult				
D_a	0.1978	0.3869	−05661	0.9616
D_{T_h}	**−4.6687**	**0.8715**	**−6.3694**	**−2.9280**
2nd instar-male adult				
D_a	−0.0416	0.2994	−0.6323	0.5492
D_{T_h}	**−0.4229**	**0.0595**	**−0.5402**	**−0.3222**

*Significant difference parameters shown in bold face.

Table 4: Parameters estimated by an equation with indicator variable for comparing type III functional response parameters of 3rd and 4th instar larvae and females of *N. arcuatus*.

Comparison stage	Estimate	Approximate SE	Approximate 95% confidence	
			Lower	Upper
3rd instar-4th instar				
D_a	−0.00760	0.00763	−0.0226	0.00743
D_{T_h}	**−0.2572***	**0.0134**	**−0.2835**	**−0.2309**
3rd instar-female				
D_a	−0.00562	0.00545	−0.0164	0.00512
D_{T_h}	**−0.1214**	**0.0116**	**−0.1443**	**−0.0985**
4th instar-female				
D_a	0.00198	0.00407	−0.00604	0.0100
D_{T_h}	**0.1358**	**0.0133**	**0.1095**	**0.1621**

*Significant difference parameters shown in bold face.

et al. [32] reported that functional responses of *Nephus ryuguus* (Kamiya) feeding on *Oracella acuta* (Lobdell) and *N. includens* feeding on *Planococcus citri* (Risso) or *Planococcus ficus* (Signoret), respectively, did not differ depending on developmental stage.

The most common functional response for coccinellids is type II and has been found in many studies: larvae and adults of *N. ryuguus* feeding on *O. acuta* [31]; larvae of *Propylea dissecta* (Mulsant) feeding on *Aphis gossypii* Glover [33]; all four larval instars and adults of *Hippodamia variegata* (Goeze) feeding on *Aphis fabae* Scopoli [34]; and 2nd instar and 4th instar larvae of *N. includens* feeding on *P. ficus* and *P. citri* [32]. In contrast, a type III functional response appears to be relatively rare among coccinellids [18, 28, 30, 35]. A predator with a type II functional response has the potential to destabilize the prey-predatory population because it causes inverse density-dependent mortality in the prey population. In contrast, a predator with a type III functional response could contribute more to regulating the density of the prey population than a predator with a type II response and is, theoretically, more capable of suppressing prey populations compared to stages exhibiting a type II response [13, 36, 37]. The three postulated mechanisms for type III functional responses in predators are as follows: (1) the concentration of a predator's hunting efforts in a high-density patch [38]; (2) switching in a multiple prey system [36]; and (3) learning [37, 39]. Our experiment with *N. arcuatus* was a short-term, single species test and so the first mechanism is most likely to be responsible for the type III functional response we observed. It is probable that these stages, by showing a type III response, have the ability to regulate prey population during outbreaks of *N. viridis* in citrus orchards.

Our results indicate that estimated attack rates did not change significantly among the different developmental stages of *N. arcuatus* with similar functional response curves observed for all stages. The attack rate determines how steeply the functional response curve rises with increasing prey density. Thus, the results revealed that the steepness did not differ among different developmental stages of *N. arcuatus* and that the different developmental stages had similar abilities to respond to increasing prey densities. In contrast, the prey handling times increased as the larval age of this predator increased, and also females had longer handling times than adult males. In other words, the 1st instar larva of *N. arcuatus* spent more time and 4th instar larvae and adult females spent less time to consume *N.*

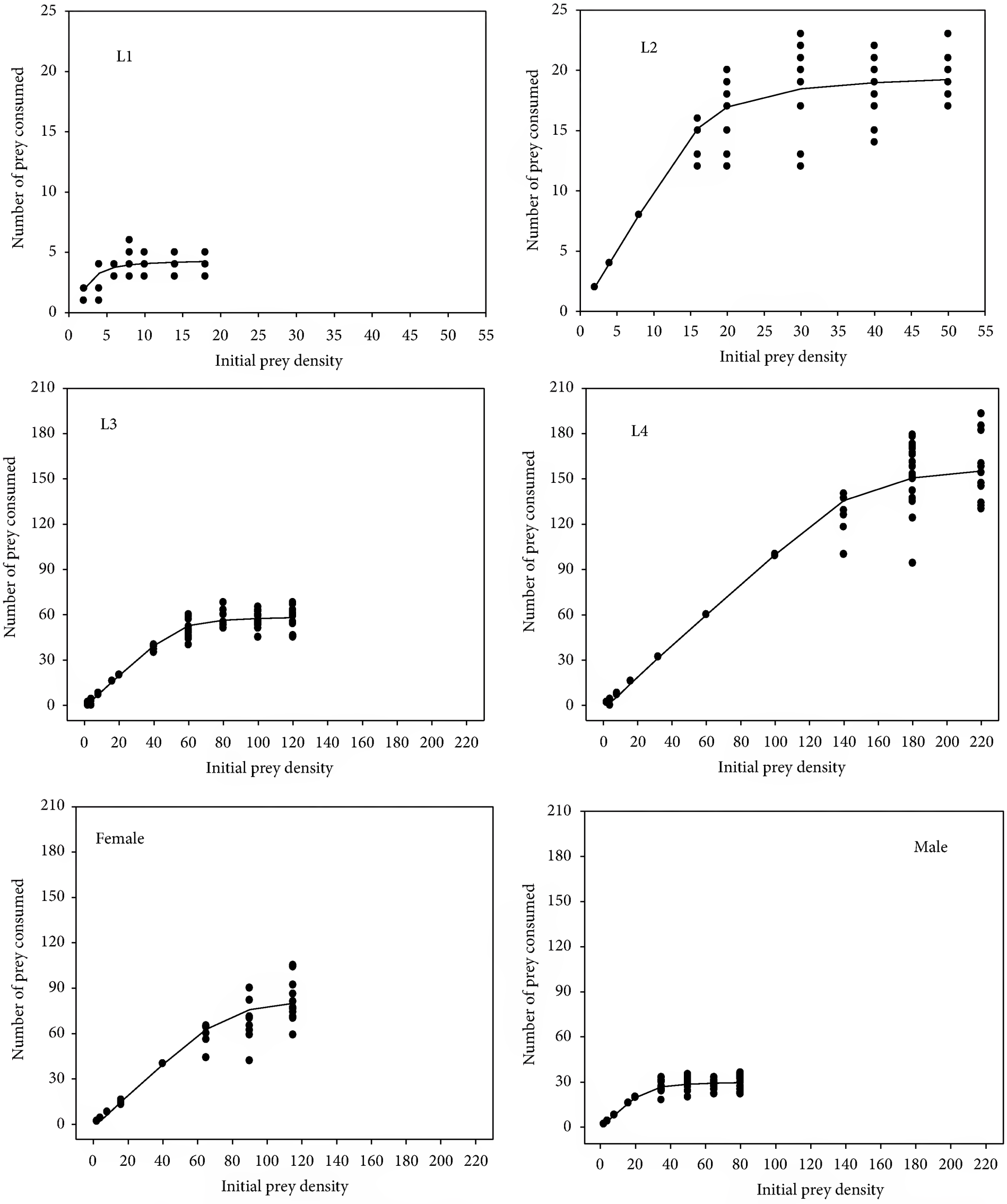

FIGURE 1: Functional responses of *N. arcuatus* to different densities of *N. viridis*. L1: 1st instar larva, L2: 2nd instar larva, L3: 3rd instar larva, L4: 4th instar larva: M: male, and F: female. Symbols are observed data and lines were predicted by model.

viridis eggs than other developmental stages. Handling time is a general term that reflects the cumulative effect of time taken during capturing, killing, subduing, and digesting prey [40]. Thus, being larger is an advantage to 4th instar larvae and adult females in subduing, consuming, and digesting more prey. Farhadi et al. [34] observed similar results for *H. variegata* feeding on *A. fabae*. Moreover, Bayoumy [28] reported that the functional response of 4th instar larvae

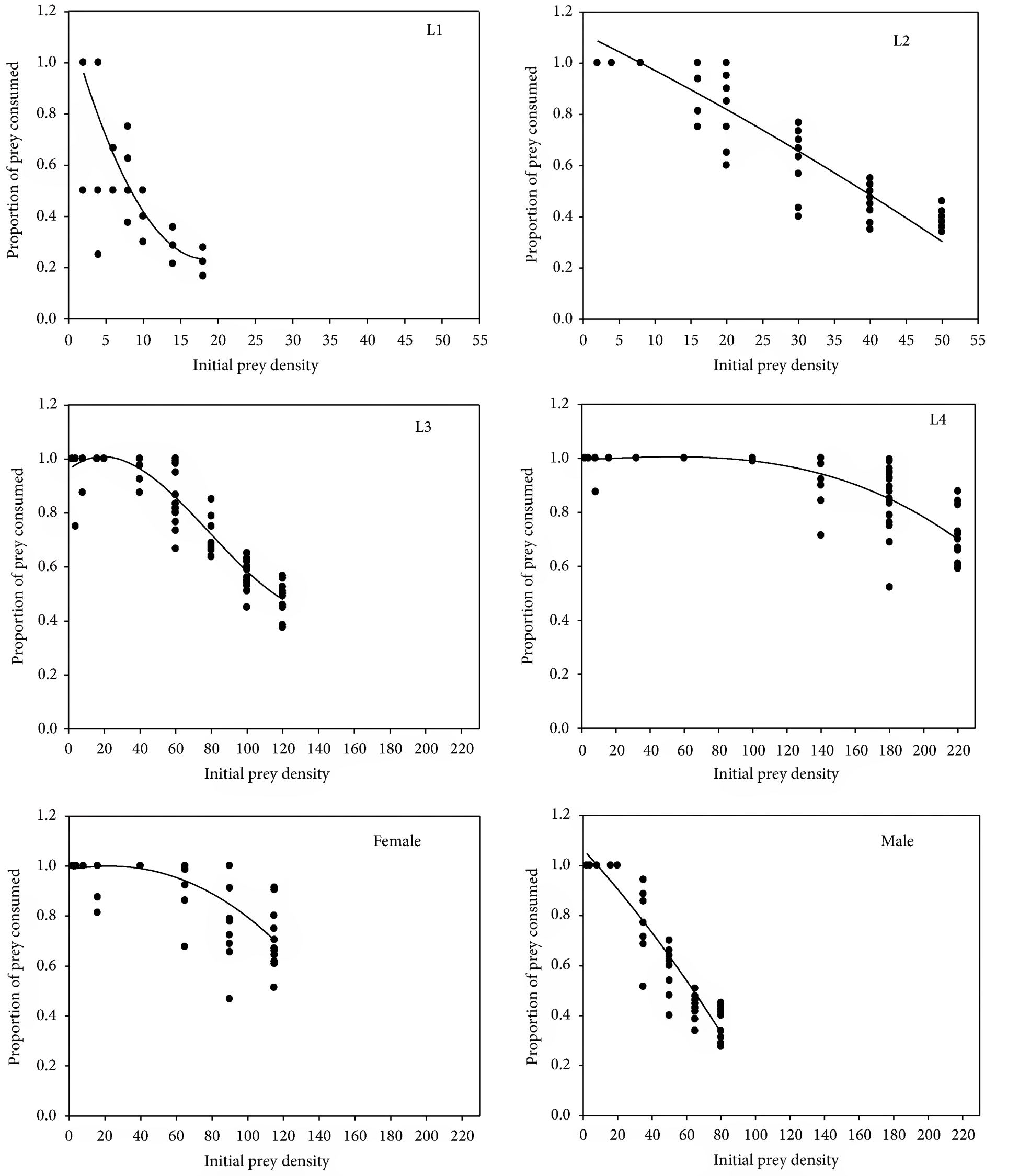

FIGURE 2: Proportion of *N. viridis* consumed by *N. arcuatus* in relation to prey density. L1: 1st instar larva, L2: 2nd instar larva, L3: 3rd instar larva, L4: 4th instar larva, M: male, and F: female. Symbols are observed data and lines were predicted by model.

and adult females of *N. includens* to *A. gossypii* differed in handling time.

The maximum predation rate per day (T/T_h) was highest for 4th instar larvae due to their greater requirements for food and energy to grow and attain the critical weight for pupation [41] or to achieve a higher search rate [28]. The second highest predation rate was for 3rd instar larvae and the third highest for adult females. These three developmental stages can, therefore, be considered as the most efficient predatory stages of *N. arcuatus*. The voracity of females may be as much as 2.6

times that of adult males. This difference may be correlated with their larger size and high nutrient requirement for egg production and oviposition [42]. The greater voracity of 4th instar larvae compared with adults is also frequently observed in other coccinellid species, for example, *P. dissecta* [33], *H. variegata* [34], and *N. includens* [28]. However, Tang et al. [31] observed that adults of *N. ryuguus* had higher predation rates than 4th instar larvae when preying on *O. acuta*.

Our results also clearly confirm that all stages of *N. arcuatus* show a high predation rate when feeding on *N. viridis* eggs. Muştu and Kilinçer [43] reported that the 4th instar larvae and adults of the related species *N. kreissli* consumed 23.5 and 47.3 eggs of *P. ficus* in 24 h, respectively. Zarghami et al. [19] observed that the males and females of *N. arcuatus* consumed 32.6 and 76.7 eggs of *P. citri* in 24 h. Thus, based on this voracity and the ability of *N. arcuatus* to survive and reproduce at temperatures around 30°C [11], this species could be efficient in controlling mealybugs in warm regions.

5. Conclusion

In conclusion, with respect to type of functional response observed and parameter estimated for all developmental stages of *N. arcuatus*, the most effective predators are, in descending order, 4th instar larvae, adult females, and then 3rd instar larvae. This laboratory study indicates that *N. arcuatus* could be an effective biocontrol agent. Specifically, a mass release of the abovementioned stages of *N. arcuatus* might provide efficient pest management especially in initial field infestations with *N. viridis* eggs. Clearly, empirical data obtained under laboratory conditions cannot be directly extrapolated to field conditions; thus further studies especially on the effect of prey age, prey size, and prey species on functional response, numerical response, long-term predation capacity, and mealybug population suppression under field conditions are needed to evaluate the possibilities for using *N. arcuatus* in inoculative/inundative biological control strategies.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Phylogenetic Analysis of the North American Beetle Genus *Trichiotinus* (Coleoptera: Scarabaeidae: Trichiinae)

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A hypothesized evolutionary history of the North American endemic trichiine scarab genus *Trichiotinus* is presented including all eight species and three outgroup taxa. Data from nineteen morphological traits and CO1 and 28S gene sequences were used to construct phylogenies using both parsimony and Bayesian algorithms. All results show that *Trichiotinus* is monophyletic. The best supported topology shows that the basal species *T. lunulatus* is sister to the remaining taxa that form two clades, with four and three species each. The distribution of one lineage is relatively northern while the other is generally more southern. The ancestral *Trichiotinus* lineage arose from 23.8–14.9 mya, and east-west geographic partitioning of ancestral populations likely resulted in cladogenesis and new species creation, beginning as early as 10.6–6.2 mya and as recently as 1.2–0.7 mya. Morphological character evolution is also briefly discussed. The limited distribution of *T. rufobrunneus* in Florida and *T. viridans* in the Midwest mainly due to urban development and widespread agriculture makes these two species of conservation concern.

1. Introduction

The genus *Trichiotinus* Casey, 1915, comprises a group of eight species that are commonly known as the hairy flower beetles due to their namesake trait of being covered in setae. The genus is placed in the tribe Trichiini Fleming, 1821, and the subfamily Cetoniinae Leach, 1815 [1]. The Trichiini currently includes 43 genera that are found nearly worldwide except for Australia and Madagascar [2, 3] with thirteen of these taxa located in the New World. In the Nearctic realm including Central America, nine genera exist including *Apeltastes* Howden, 1968, *Archedinus* Morón and Krikken, 1990, *Dialithus* Parry, 1842, *Giesbertiolus* Howden, 1988, *Gnorimella* Casey, 1915, *Iridisoma* Delgado-Castillo and Morón, 1991, *Paragnorimus* Becker, 1910, *Trigonopeltastes* Burmeister, 1840, and *Trichiotinus* Casey, 1915 [4, 5].

Trichiotinus is widespread in North America (Figure 1) with species distributed from Florida to Texas to southern Canada including Nova Scotia in the east to and as far north as the Northwest Territories in the west [3, 6]. While several species are widespread, others are relatively restricted including one species found mainly in Texas, another endemic to part of central Florida, and a third found in a small portion of the Midwest through to southern Ontario [7]. Adults are beautifully patterned and colored yet are thought to be harmful to flowers because they eat pollen and petals [8]. But due to their hirsute bodies, they certainly may act as successful pollinators [6]. Larvae are known to feed upon various types of dead hardwood.

The most obvious morphological characters uniting the species include the body dorsally and ventrally setose, the elytral margin bowed downward below and behind the humeral angle, the elytra with two raised intervals, and the presence of two transverse cretaceous (chalky white) bands in most but not all species [3]. Among taxa, there appear to be few morphological variations that can help hypothesize evolutionary history within the genus. A phylogenetic analysis was done using as many morphological characters that could be discovered, as well as molecular data from two genes. Furthermore, we briefly explore character evolution

Table 1: List of taxa used in the analysis, their collection origin, and accession numbers for DNA sequences on GenBank.

Taxon	Locality	CO1	28S
Trichiotinus affinis (Gory & Per.)	Kentucky, Warren Co.	KX132104	KX151980
Trichiotinus affinis (Gory & Per.)	Quebec, Gatineau	KX132105	KX151981
Trichiotinus assimilis (Kirby)	Ontario, Muskoka	KX132107	KX151983
Trichiotinus bibens (Fab.)	Kentucky, Warren Co.	KX132111	KX151987
Trichiotinus lunulatus (Fab.)	Texas, College Station	KX132112	KX151988
Trichiotinus piger (Fab.)	Illinois, Sumner	KX132108	KX151984
Trichiotinus rufobrunneus (Casey)	Florida, Tallahassee	KX132109	KX151985
Trichiotinus texanus (Horn)	Texas, College Station	KX132110	KX151986
Trichiotinus viridians (Kirby)	Illinois, Sumner	KX132106	KX151982
Outgroups			
Gnorimella maculosa (Knoch)	Quebec, Gatineau	KX132113	KX151989
Trigonopeltastes delta (Forster)	Kentucky, Warren Co.	KX132114	KX151990
Osmoderma eremicola (*Knoch*)	Tennessee, Portland	KX132115	KX151991

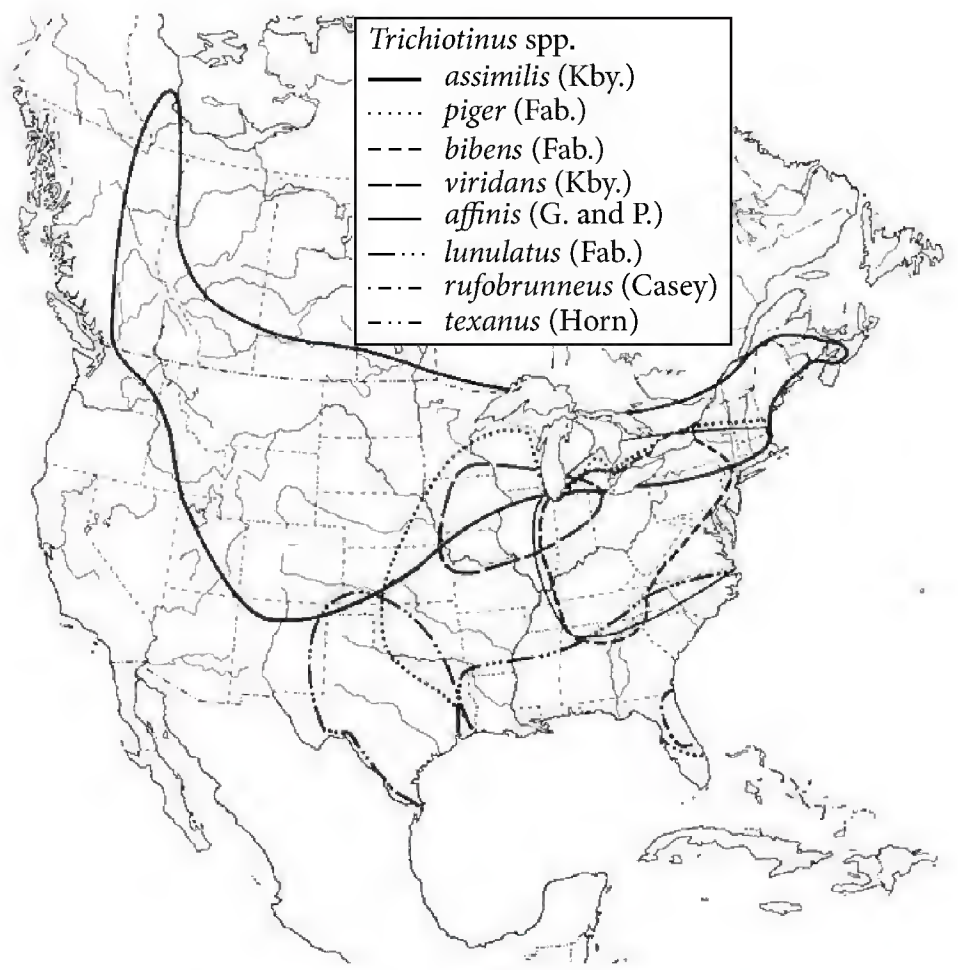


Figure 1: Distributions of the eight species of *Trichiotinus* from [3] (used with permission).

and the biogeography of the group and hypothesize the dates of cladogenesis based on CO1 divergence.

2. Materials and Methods

2.1. Sampling. Specimens were collected in various localities within the USA and Canada (Table 1). The molecular analysis used fragments of DNA sequence data from CO1 and 28S genes (ca 800 bp fragment of mtDNA cytochrome oxidase subunit 1 (COI) and ca 560 bp of D2 loop of nuclear 28S rRNA). While the CO1 gene is a standard gene used in phylogenetic studies, the nuclear 28S rRNA gene is a mosaic of highly conserved and variable regions [9] and has also proven to be useful for resolving relationships in numerous insect groups (e.g., [10, 11]), including studies of beetles (e.g., [12, 13]).

2.2. DNA Sequencing. DNA was extracted using the Omega Bio-Tek E.Z.N.A. Insect kit. Muscle tissue was removed from the pro- and mesothoracic regions and ground up to improve the extraction of DNA. Sequences were amplified by PCR using combinations of published primers [14] and 5 Prime master mix was used in the amplification process. Typical PCR cycles for COI consisted of an initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 30 seconds, 46°C for 30 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes. The PCR reaction for 28S was similar except for an annealing temperature of 55°C. Amplification of the 16S gene was attempted but the results were very poor in early tests and work on this gene was discontinued.

PCR products were purified prior to sequence reactions and sequenced using ABI dye-terminator v3.1, following the standard protocol on an ABI3130 sequencing machine. DNA sequences were edited in Geneious version 7.1.4. Sequences were deposited in GenBank with accession numbers listed in Table 1.

2.3. Morphological Data. Morphological data was acquired by soaking specimens in lactic acid to macerate tissues before dissection. Various individual body parts were examined on slides in glycerine or dry to discover characters and states. Mouthparts were examined and found to be so similar that no useful character states from them were discovered. Some characters used to define the genus such as two protibial teeth were found to be uninformative and were excluded. Two characters were uninformative but each has three states (char. 10 and 14) and are included in the analysis. Characters and states for each taxon are listed in Table 2 and were found from the head, pronotum, elytra, pygidium, and ventrites with the descriptions as follows:

(1) Elytral third and fifth intervals: strongly convex (0); feebly convex to flattened (1).

Table 2: List of taxa and their morphological character states used in this study. Note that *Trigonopeltastes* in character seven is coded as having both states as indicated within the parentheses.

Osmoderma eremicola	-141-11	2113712??110
Gnorimella maculosa	-121-11	0302611??111
Trigonopeltastes delta	112112(0/1)	0222500??001
Trichiotinus_affinis_KY	1120000	110110010000
Trichiotinus_affinis_QC	1120000	110110010000
Trichiotinus_assimilis	1120000	110110011000
Trichiotinus_bibens	1000101	110041011000
Trichiotinus_lunulatus	1000101	100031011000
Trichiotinus_piger	0110100	100100001000
Trichiotinus_rufobrunneus	0110100	110100001000
Trichiotinus_texanus	0130100	100100001000
Trichiotinus_viridans	1120000	100120010000

(2) Head and pronotum color: bright metallic green (0) or not (1).

(3) Elytra color: (0) bright metallic green; (1) light brown; (2) a combination of black and brown (may be some greenish reflections); (3) black; (4) dark brown.

(Note that although *T. bibens* appears brownish underneath a bright metallic green, the species is classified as having state 0.)

(4) Elytral lateral margin below humeral angle: abruptly deflexed down and outward (0) or smoothly rounded (1).

(5) Elytral fourth interval: sparsely punctate (0); distinctly puntate (1).

(Note that *Osmoderma* and *Gnorimella* have elytra lacking distinct intervals and are coded as inapplicable.)

(6) Pygidium shape: approximately round with the width ~equal to the length (0); transverse, wider than long (1); narrow, longer than wide (2).

(7) Elytra: obliquely transverse narrow white bands present (0); bands absent (1).

(Note that *Trigonopeltastes delta* does not have bands present but at least one species does (*T. sallaei*). Therefore this taxon is coded as having both states present.)

(8) Pygidium cretaceous patch: covering surface (0); on lateral edges only (1); absent (2). (This character in *Gnorimella* is dimorphic where females have the patch covering the surface and males have both lateral and a central longitudinal oriented patch. Further, some males appear to have no cretaceous patch at all. This character was coded for females. Some species of *Trigonopeltastes* have a patch similar to that seen in *Trichiotinus* but were coded as seen in *T. delta* typically with the entire surface covered.)

(9) Ventrite cretaceous band: on 5th laterally (0); absent (1); covering entire surface on 1st–5th (2); covering lateral surface on 1st–5th (3).

(10) Pronotal setae: present, erect, and dense (0); absent (1); setae recumbent and on outer edge in a line in triangular pattern (2).

(11) Elytra: dull or opaque area on lateral declivous portion: absent (0); present on posterior 2/3 (1); present on entire surface (2).

(12) Male paramere teeth: 2 teeth at apex (0); 1 tooth at apex (1); no teeth and apex tapered (2); no teeth and apex abruptly expanded and rounded (3); no teeth and apex gradually expanded and pointed (4); 1 tooth ~medially (5); no teeth and broadly curved at apex (6); no teeth and narrowly curved at apex (7).

(13) Short cretaceous (i.e., chalky white) longitudinal band just posterior of scutellum: present (0); absent (1).

(14) Scutellum shape: elongate triangular and sides slightly rounded (0); broadly triangular and sides rounded (1); elongate triangular and sides straight (2).

(15) Female genitalia, dorsal view: stylus elongate and transverse (0) or broader, blunt, and/or obliquely positioned (1).

(16) Female genitalia, dorsal view: apical tooth short and small (0) or not (1).

(17) Metatarsal length: distinctly longer than metatibia (0) or shorter than metatibia (1).

(18) Elytral striae: present (0); absent (1).

(19) Elytral surface: in part smooth and shiny (0); velvet-like throughout surface (1).

2.4. Analyses. All sequences were assembled and edited by eye using the program Geneious, version 7.1.4 (Genecodes Corp., Ann Arbor, MI). Alignment of the CO1 and 28S data sets was done using Muscle, version 3.8.425 [15], with the default parameters including 8 iterations, max. number of trees to build = 1, and optimization = anchor.

The data matrix was first constructed using WinClada [16]. Parsimony analysis was done using Nona and TNT [17, 18]. All characters were coded as unordered, and the matrix was analyzed with equal weights. The search was implemented using the following parameters, for example, in TNT: hold 10 000, hold/50, Mult * 1000 (random addition sequence, 1000 replicates and TBR branch swapping). Character evolution and node support were done using WinClada.

JModelTest version 2.1.7 [19] was used to determine the best model to use in the Bayesian analyses. For the 28S sequence data the best model was determined to be HKY + I while for the CO1 the best model was TIM2 + G. As the latter is not a model available in Mr. Bayes, the next best available, a GTR + G model of nucleotide substitution was used.

Bayesian analyses were executed in the programs MrBayes, version 3.1 [20]. For Bayesian analyses, a relative burn-in of 25.0% and 1,000,000 generations were used as well as the other default values. For the 28S analysis,

the commands lset nst = 6 and rates = propinv were used while the CO1 analysis used lset nst = 6 and rates = gamma. The morphological analysis used the standard morphological model with lset rates = gamma, coding = variable, prset symdirihyperpr = fixed (infinity), and ratepr = variable. These same commands were used in the total evidence partitioned data analysis. The standard deviation of split frequencies between the two simultaneous analyses decreased below 0.01 within 330,000, 265,000, 505,000, and 10,000 generations for the CO1, 28S, morphology, and concatenated total data, respectively, and visual analysis of trace plots of the likelihoods of sampled trees was also examined to determine when the MCMC chains had reached stationarity.

Clade support based on the majority rule values was discovered in Mr. Bayes while node support was evaluated in WinClada. Bootstrap and jackknife values were calculated using 1000 replications and 10 search replications with one starting tree per replication and without tree bisection-reconnection (TBR). Each data set was explored individually and in combination as total evidence with both parsimony and Bayesian analyses. Trees were rooted between the ingroup and the three outgroups.

2.5. Molecular Dating. The application of a global molecular clock has been shown to be difficult both methodologically and philosophically [22, 23]. In addition, its applicability is difficult to assess. Nevertheless, the COI region is extensively used due to its relatively consistent rate of change among lineages [24] and the idea that it is better to have a possibly poor estimate rather than no estimate at all. Hence this partial CO1 sequence was used herein for dating branch divergences.

The most used calibration in insects assumes a rate of 2.3% sequence divergence per one million years [25, 26]. Based on the published rates for other groups of beetles [27–31] the clock with the rates of 0.0075 and 0.012 (Beast 1.4.8 [32]) was used to calculate the time to the most recent common ancestor (MRCA) for each clade. To account for rate heterogeneity among sites, a gamma distribution was used. The Yule speciation model generated a tree using a lognormal relaxed clock. Two independent runs of the MCMC for five million generations (sampling every 5,000 generations) were performed for each clock rate. Burn-in was set to 10%. Tracer 1.3 [33] as used to evaluate the convergence of the chains in both runs.

3. Results

We successfully obtained DNA sequences for the two target gene regions for all 12 of the ingroup and outgroup taxa included in this study. CO1 data ranged from 799 to 801 bases in length with 147 informative characters. 28S sequence data was generally about 560 bases with 36 informative characters. Two taxa had significantly shorter 28S sequences due to poor quality sequencing and included *Gnorimella* (346 bases) and *T. assimilis* (368 bases).

Trichiotinus is strongly supported as a monophyletic genus using either parsimony or Bayesian analyses (Figures 2–4). Based on the included outgroups, the most likely sister genus seen in all but two analyses is *Trigonopeltastes*; in the Bayesian CO1 analysis *Gnorimella* is the sister clade (Figure 3(a)) while in the parsimony 28S analysis one of two trees shows *Gnorimella* + *Trigonopeltastes* as the sister clade (strict consensus in Figure 3(d)). Within the ingroup, *Trichiotinus lunulatus* is the sister to the other seven species in both parsimony and Bayesian total evidence analyses as well as the topologies using only the CO1 data. Clades supported in most analyses include ((*T. piger* + *T. rufobrunnea*) + *T. texanus*) and usually + *T. bibens* (Figure 2, clade 1) as well as (*T. affinis* + *T. viridans*) and usually + *T. assimilis* (Figure 2, clade 2). All clades within *Trichiotinus* have strong support in the total evidence analyses (Figure 2) giving one confidence in this hypothesis of evolution. All support levels are greater than 89% in the Bayesian topology while in the parsimony analysis the support is above 92/95 for the bootstrap and jackknife values, respectively, with the exception of clade 1 (Figure 2) with values of 52/50 supporting this node. The parsimony total data analysis produced a single tree of 609 steps and CI = 71 and RI = 57.

Parsimony analysis of the morphological data alone produces a single 40-step tree (CI = 90, RI = 87) that is similar to the total evidence topology except that *T. bibens* + *T. lunulatus* together form a clade that is sister to the other *Trichiotinus* species instead of *T. lunulatus* as sister to all remaining species (Figure 4). Additionally, *T. piger* is sister to *T. texanus* instead of *T. rufobrunneus*. In contrast to parsimony, the Bayesian morphological topology is relatively unresolved with a hexatomy; the only clades within *Trichiotinus* that appear are a trichotomy consisting of *T. texanus*, *T. rufobrunneus*, and *T. piger* and a second clade composed of *T. lunulatus* + *T. bibens*.

The CO1 data analyzed using parsimony (Figure 3(b)) is identical to that found with total evidence analyses. In contrast, the Bayesian topology is similar but less resolved with a trichotomy created via the unresolved position of *T. bibens* (Figure 3(a)). Topologies from the 28S data in either Bayesian (Figure 3(c)) or parsimony analyses (two trees discovered, strict consensus in Figure 3(d)) strongly support clade 1, but clade 2 is disrupted in part by the unusual placement of *T. assimilis*. This may be due to the reduced sequence length for this species due to poor quality and the necessity of greater editing; while other species generally had about 540 bases, *T. assimilis* had only 368 bases included in the analysis. One should also note that only 36 bases were informative for the full length of this sequence. One other unusual aspect to this single gene analysis is the shift to a basal position of both *T. affinis* and *T. viridans* and is in stark contrast to their placement in all other analyses.

4. Discussion

The genus *Trichiotinus*, based on the total evidence topology, is composed of three main lineages; a single species, *T. lunulatus*, is sister to all other species and these in turn form two sister clades (labeled 1 and 2) as seen in Figure 2. Morphologically, the eight species within the genus *Trichiotinus* are quite similar, with only what might be considered minor differences. But at least in North America, the genus is unique

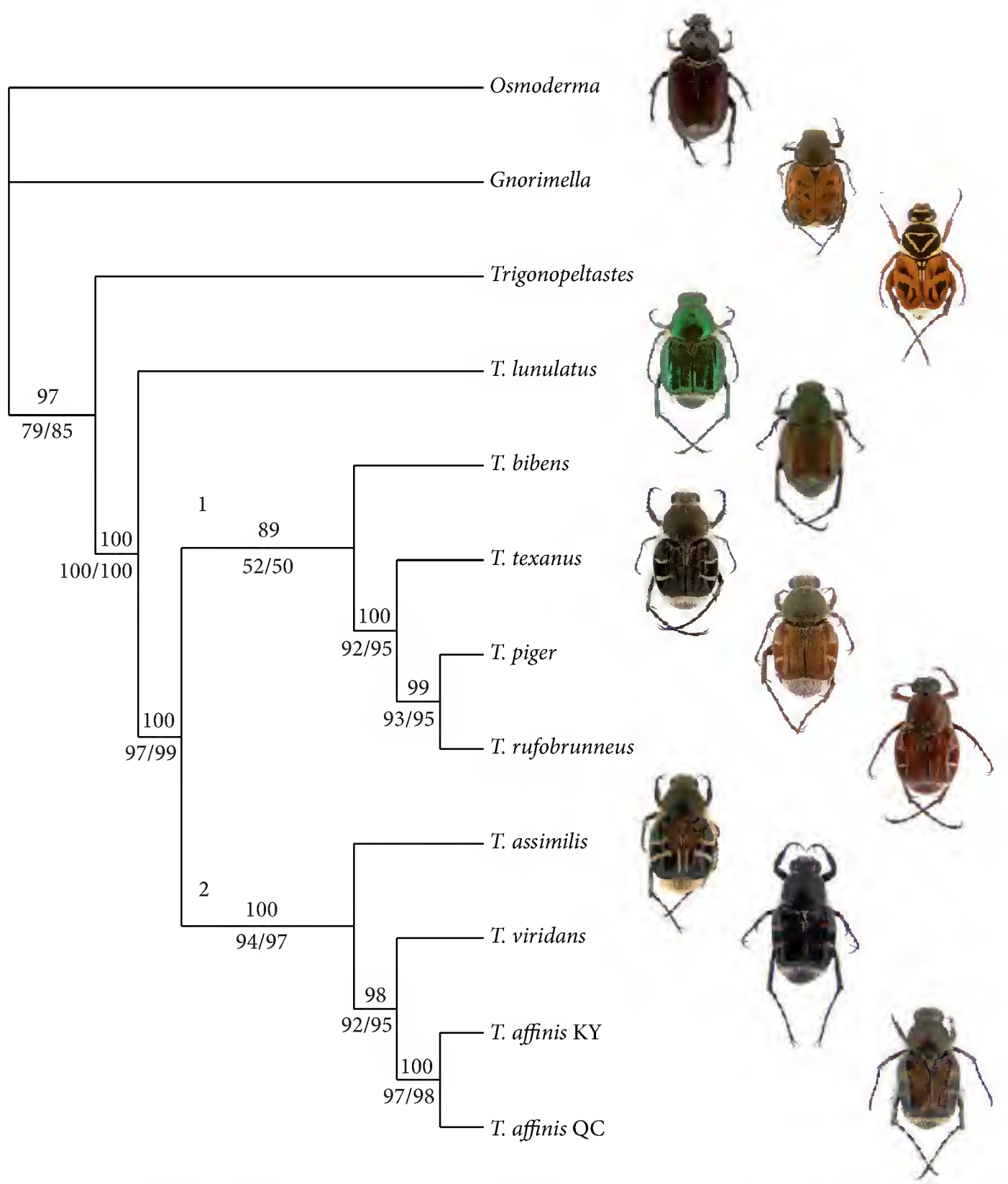
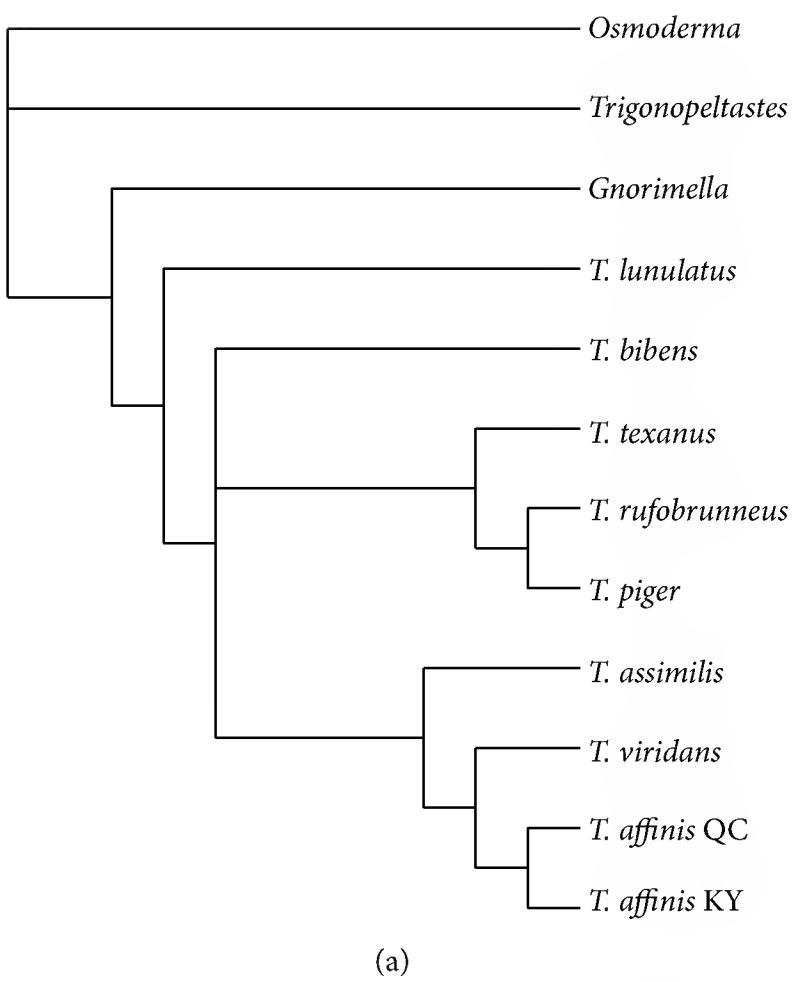


FIGURE 2: Topology of *Trichiotinus* found using both parsimony and Bayesian analyses and with all three data sets combined (CO1, 28S, and morphology) as well as a parsimony analysis using just the CO1 data. This is considered the best supported topology for the genus. Clade support values from the Bayesian analysis (above) and bootstrap/jackknife values (below) from the parsimony analysis are shown adjacent to each node, respectively. The two major clades discussed are labeled as 1 and 2 and the two included *T. affinis* sampled from Kentucky and Quebec are indicated.

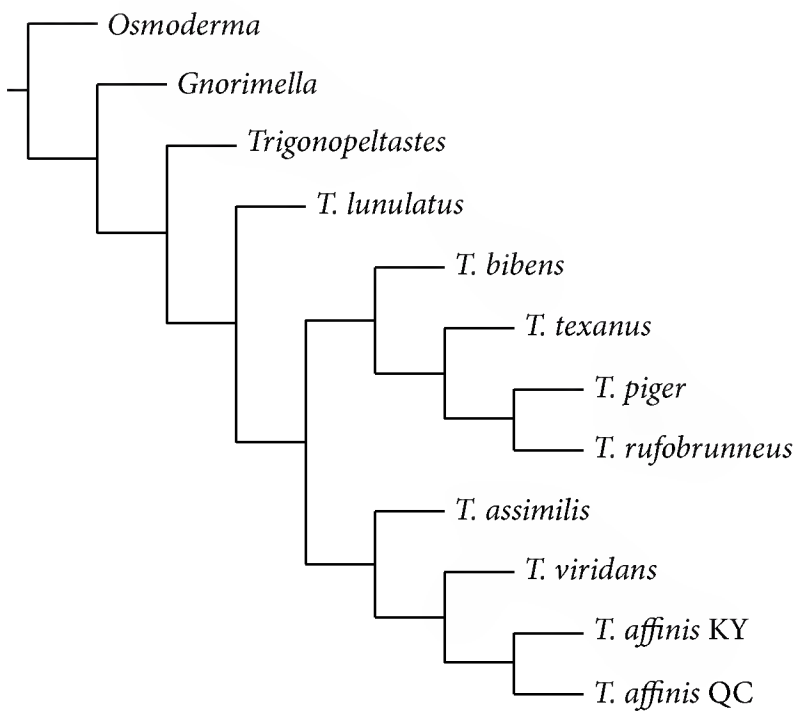
and distinct from other genera by the presence of an elytral lateral margin below humeral angle that is deflexed down and distinctly projected outward (character 4, state 0). Other characteristics used to define the genus from closely related taxa are not as useful, including the pygidium cretaceous patch on lateral edges only (character 8, state 1). While this is different from the included *Trigonopeltastes delta*, other species in this genus as well as other genera also have a similar shaped pygidial patch (see [3]).

The sister relationship of *T. bibens* + *T. lunulatus* as seen in the tree based upon morphological tree (Figure 4) is based on states that use the bright metallic green color of the head + pronotum and the elytra. Hence this relationship should be considered weakly supported and further is not seen in the molecular or total evidence topologies. This clade is sister to the lineage that is supported by a single character, the presence of obliquely transverse white bands on the elytra (character 7, state 0). Based on the total evidence tree, this state either evolved twice within both clade 1 and clade 2 (Figure 2) with the selection pressure perhaps due to becoming morphological similarity to bees or (and less likely) was lost in *T. bibens*.

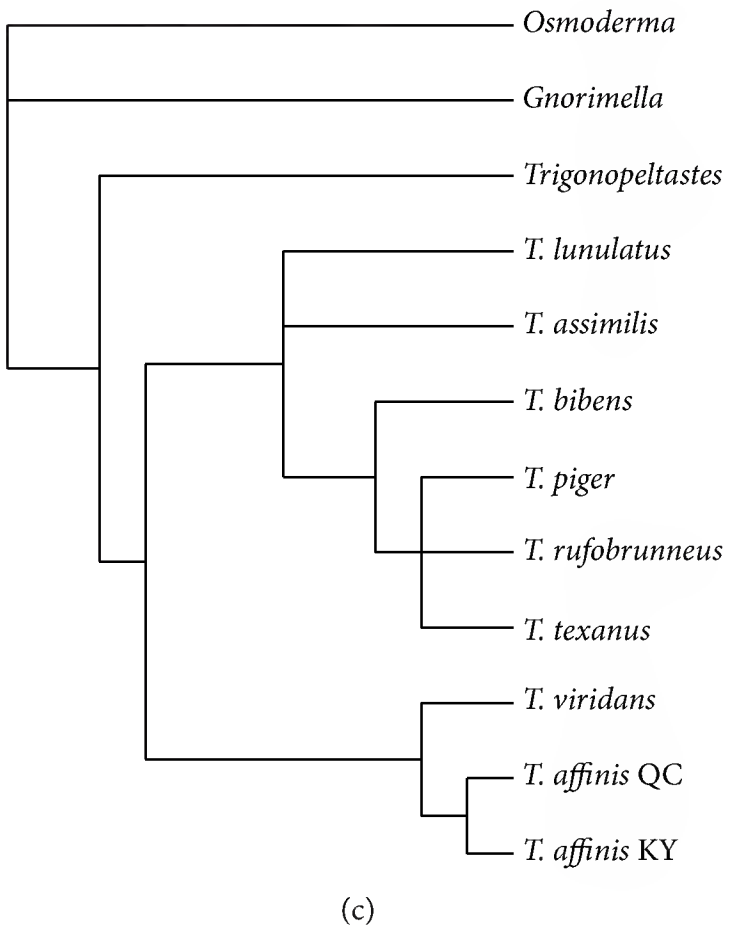
4.1. Distribution. All species of *Trichiotinus* are found primarily in the mid and eastern parts of North America although one species (*T. assimilis*) extends into the western states within the mountain time zone and north into the territories of northwestern Canada (Figure 1). Species appear to fall into two main geographic patterns that may reflect some degree of temperature range preference or tolerance, as



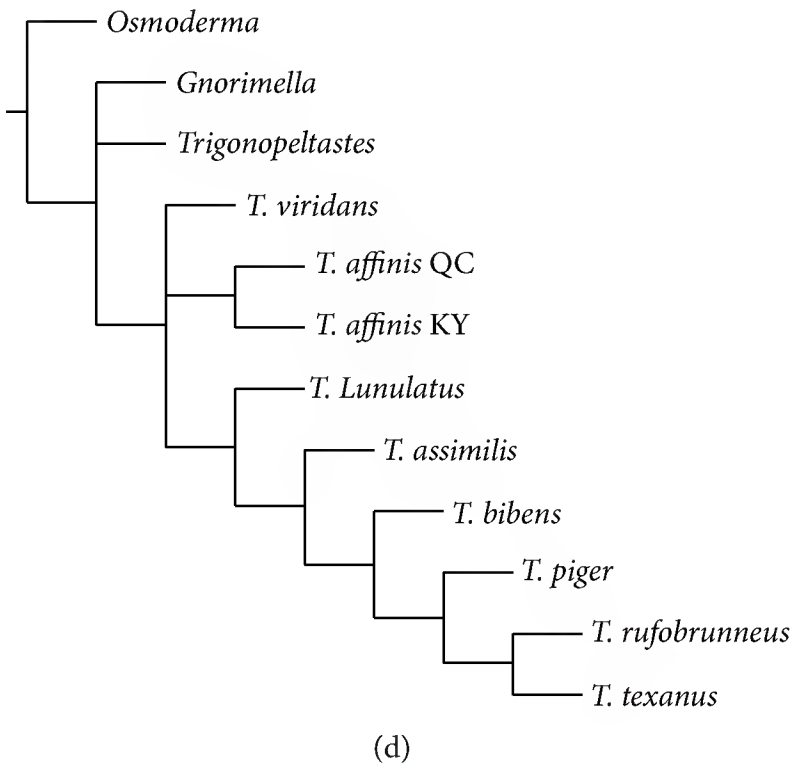
(a)



(b)



(c)



(d)

Figure 3: The Bayesian and parsimony analyses using molecular data. (a) Bayesian analysis using the CO1 data. The tree is similar to that found with the total evidence except for an unresolved trichotomy near the base of the topology; (b) parsimony analysis using CO1 data. The tree is identical to that found with the total evidence; (c) Bayesian analysis using 28S data; (d) Parsimony analysis using 28S data, strict consensus of two topologies.

no obvious geographical barriers exist. Clade 2 (*Trichiotinus assimilis*, *T. viridans*, and *T. affinis*) has a distribution largely in the northern and central part of the USA and appears to have a colder temperature tolerance. All other species (*T. lunulatus* and clade 1) are restricted to the middle and southern parts of the Midwest and eastern USA reflecting a higher temperature tolerance.

Adults are good fliers and forage on a variety of flowers while larvae are known to feed on various species of decaying hardwoods. Hence the restricted distributions of some species are somewhat puzzling. In particular, *T. viridans* has an odd distribution in the Midwest but that may reflect an association with the northern half of the mid-western oak-savanna habitats (see [34] for oak-savanna distribution figure). *Trichiotinus rufobrunneus* also has a very small distribution within Florida and again is likely restricted to oak scrub habitats.

4.2. Evolution in the Genus. Although there are issues with time estimations using CO1 data, our conservative approach

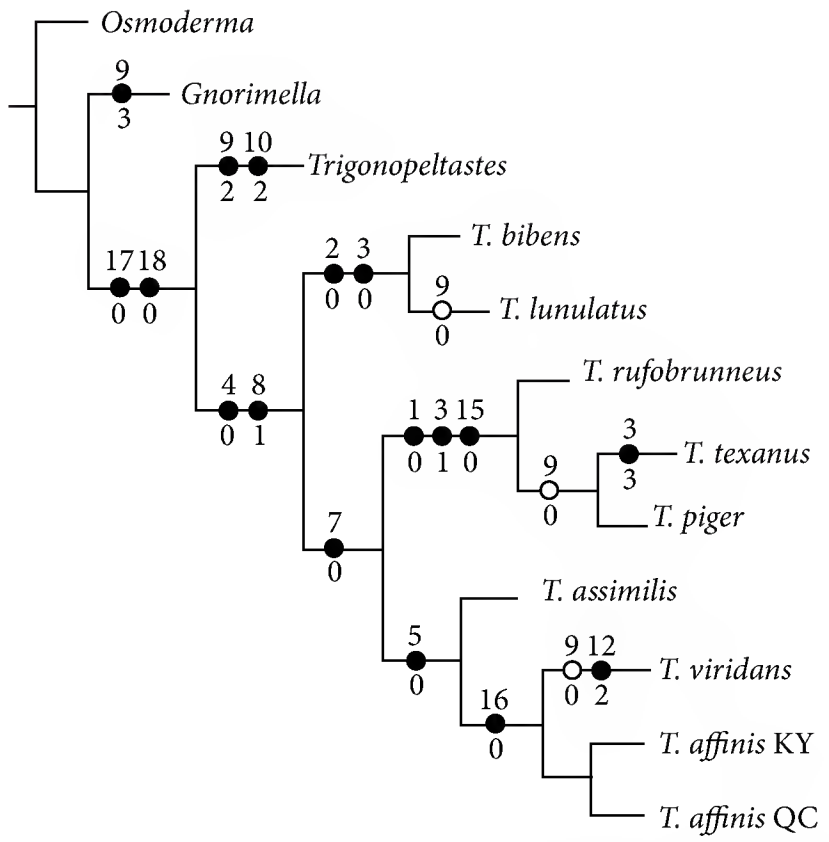


FIGURE 4: Topology based on morphological data from the parsimony analysis showing clade support (characters above and character states shown below). Solid black dots indicate character states without homoplasy. The Bayesian analysis of morphology was similar but much less resolved.

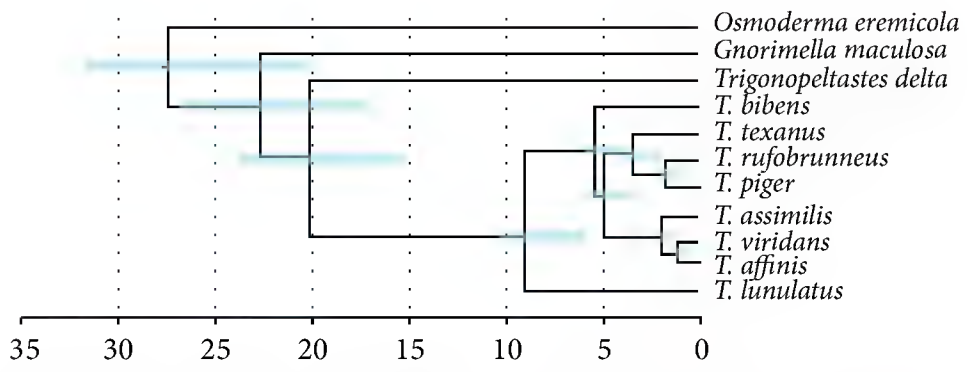


FIGURE 5: Tree showing divergence via the molecular clock. Dates are millions of years earlier and include the probable minimum and maximum age of cladogenesis.

supports the origin of this genus in the early Miocene (14.9–23.8 mya) (Figure 5). The sister clade most often appearing in this study, *Trigonopeltastes*, is a mainly a Mexican and Central American Neotropical genus. However, as no Old World representatives were included as outgroups, it is possible that the sister clade is from that region. Howden [3] speculates that *Trichius* is a possible sister lineage, but that perhaps a different Asian clade, of which he only knew from descriptions, might also be likely. If the genus does have evolutionary ties with an Asian lineage, there were strong links between Asia and North America from the late Paleocene to the middle-late Oligocene, when disjunction arose between North America and Asia [35]. Hence, the split of the common ancestor into two lineages, with one being the ancestral *Trichiotinus* species, may be related to the Middle Miocene Climatic Transition (MMCT) when temperatures on the planet began a rapid decrease [36]. More complete phylogenetic study will be needed to better hypothesize the sister clade but was beyond the scope of this study.

The earliest split in the *Trichiotinus* clade occurred in the late Miocene 10.6–6.2 mya (Figure 5). One lineage resulting from this event is represented by *T. lunulatus* while the second includes the seven remaining species. Based on Figure 2 (clade 1 and clade 2), the next cladogenic event resulted in two major lineages. Although only clade 2 appears as monophyletic in the molecular clock topology (Figure 5), for clade 1 the origin is estimated at 6.3–3.6 mya, reflecting the maximum age when *T. bibens* of clade 1 split from the remaining six taxa and the minimum age when the remaining three taxa of clade 1 separated from clade 2.

Clade 1 includes all species with a more southern distribution compared to clade 2, although *T. piger* confounds this somewhat with a distribution extending north into southern Canada. Nonetheless, possibly due to a cooling climate, this lineage may have been isolated in the southwestern part of the current range. In contrast, clade 2 may have been isolated in the southeast. Without the ability to shift further south due to a possible distribution on the Florida peninsula (even with the continental shelf exposure during maximum glaciation events), this lineage may have become by necessity more tolerant of colder temperatures that may be reflected in a generally more northernly present day distributions for all of the species in clade 1. All three species (*T. affinis*, *T. assimilis*, and *T. viridans*) are currently found no further south than approximately 34° north latitude in northern Alabama. *T. assimilis* in particular appears to be more cold tolerant than any other species as evidenced by a distribution as far north as the territories of Canada (Figure 1).

4.3. Further Cladogenesis. All remaining divergence and speciation within the genus occurred no earlier than four million years to as recently as 700,000 years earlier (Figure 5). During the late Pliocene about 3.6 mya the climate deteriorated and became more variable through and into the Pleistocene glacial/interglacial cycles [37]. About 3 mya, large ice sheets appeared in the high latitudes of the Northern Hemisphere and continued to grow rapidly for another million years. Additionally, the climate became more variable as seen in the 41,000 yr obliquity cycles due to axial tilt shift in the Earth. Hence the cause of additional cladogenesis in *Trichiotinus* is most easily attributed once again to successive glaciation events dividing ancestral populations into western and eastern blocks long enough for speciation and reproductive isolation to occur.

At least some evidence suggests that there was a broad band of warm mixed (temperate) forest/woodland across central North America during the middle Miocene [38]. Glaciation would have destroyed habitat and shifted these forests much further south than they currently exist (Figure 6). And with ice extending furthest in the Midwest compared to the eastern and western parts of the USA, the hardwood tree species needed by *Trichiotinus* may have been divided into western and eastern populations.

Trichiotinus is dependent upon decaying hardwoods as larval food including oak [6]. Jackson et al. [39] present evidence for a split in distribution of oaks (*Quercus* spp.) during the most recent glacial maximum on either side of the Mississippi drainage and may be indicative of the effects

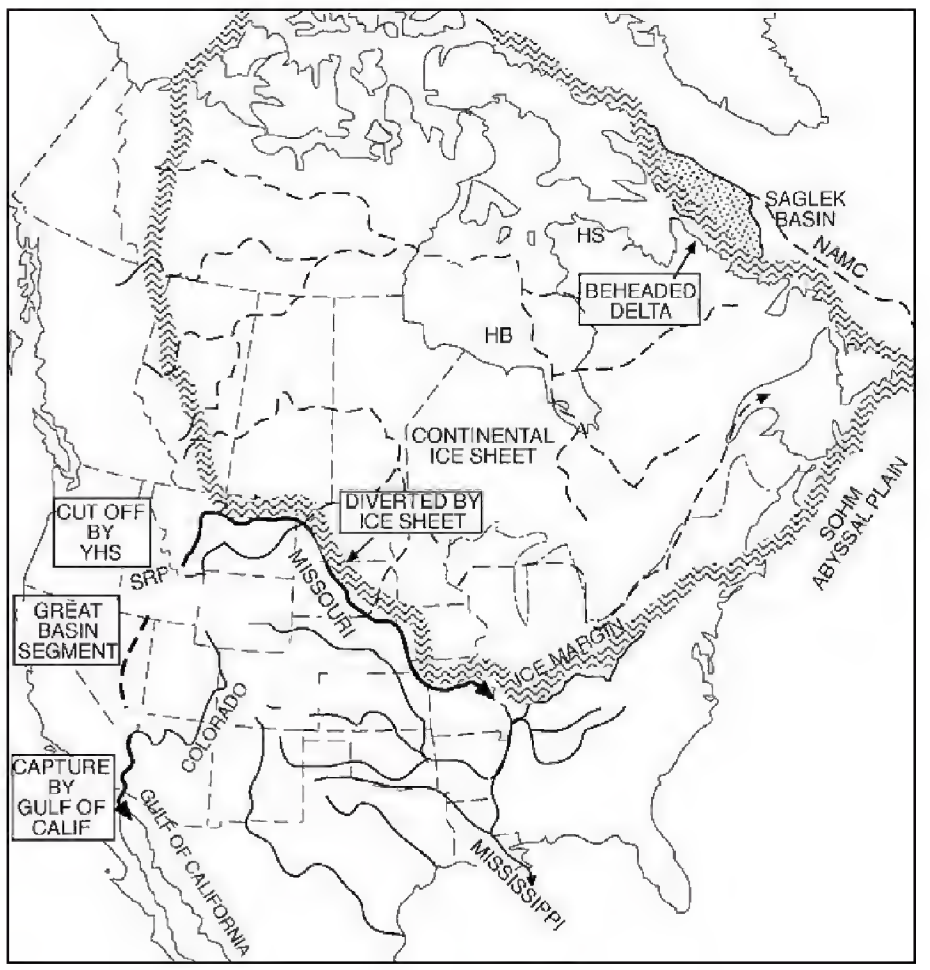


FIGURE 6: Maximum extent of the last Pleistocene glaciation in North America. Modified from [21].

of earlier glacial maxima as well. P. A. Delcourt and H. R. Delcourt [40, 41] also postulated the presence of spruce (*Picea glauca*) forests in the Lower Mississippi Valley. This extension south of these more cool adapted forests all the way to the gulf coast that divided the hardwood forests into eastern and western blocks was thought to be due to glacial meltwater flow creating a cooler climate locally [42].

During the Pre-Illinoian Stage, an interglacial event with higher sea levels likely isolated a population of *T. piger* in Florida that speciated into *T. rufobrunneus* sometime between 1.2 and 2.0 mya. Perhaps similar to the Florida Scrub Jay, both species appear to be largely restricted to xeric oak scrub and scrubby pine flatwoods habitats in Florida. Lastly, the Nebraskan Glaciation event that occurred from 780,000 to 900,000 years earlier [43] correlates closely with a dated cladogenesis event in *Trichiotinus* of 700,000 to 1.2 mya. The western population evolved into *T. viridans* while the eastern population is now recognized as *T. affinis*.

4.4. Conservation. Future studies or perhaps some degree of population monitoring should be considered to address potential conservation concerns for *T. rufobrunneus* due to their limited distribution in Florida as well as *T. viridans* that is found only in the "corn and soybean deserts" of the upper Midwest, where unfortunately little natural habitat remains. Distribution records are largely based on data from 1935 [6] and it is likely that a reduction in distributions of these and other species has occurred since this time. Future studies could map out current distributions as well as abundances; at least in Kentucky, beetles can be difficult to find in what appears to be good forest habitat for both larvae and flowers used for adult feeding for unclear reasons (Philips, unpublished).

Disclosure

The current address of M. Callahan is Department of Biology, California State University, Monterey Bay, Seaside, CA 93955, USA.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Capture of Nontarget Flies (Diptera: Lauxaniidae, Chloropidae, and Anthomyiidae) on Traps Baited with Volatile Chemicals in Field-Crop Habitats

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Volatile chemicals increased trap catch of flies from the families Lauxaniidae [*Homoneura bispina* (Loew) and *Camptoprosopella borealis* Shewell], Chloropidae (*Olcella* sp.), and Anthomyiidae (*Delia* spp.) in field crops. With lauxaniids, baiting with 2-phenylethanol on cotton-roll dispensers increased catch of *H. bispina* in two corn plot tests, and methyl salicylate increased trap catch in one test. Traps baited with methyl salicylate increased the catch of *C. borealis*. When using plastic-sachet dispensers, traps baited with methyl salicylate caught more *H. bispina* than ones baited with 2-phenylethanol, whereas traps baited with 2-phenylethanol caught more *C. borealis* than those with methyl salicylate. For chloropids, traps baited with 2-isopropyl-3-methoxypyrazine greatly increased catch of *Olcella* flies in corn and soybean. With anthomyiids, catch of male *Delia* flies in wheat increased with 2-phenylethanol on cotton rolls and with either 2-phenylethanol or methyl salicylate using plastic dispensers. In soybean, 2-phenylethanol formulated on cotton rolls or in plastic dispensers increased catch of male *Delia* flies, but methyl salicylate did not affect trap catch. Trap catch of female *Delia* flies did not vary among chemicals. In another test in soybean, trap catch of both male and female *Delia* flies was greater with 2-phenylethanol than with other volatile chemicals.

1. Introduction

Chemical cues are important to the basic life history of insects in activities such as locating food, finding mates, and recognizing suitable habitat [1]. However, the chemical ecology of many insects is still poorly understood. This is particularly true for noneconomic species, as methodical studies on their chemical ecology are often lacking.

Although systematic, hypothesis-driven research can produce novel information about insect chemical ecology, new knowledge may sometimes arise from unexpected sources [2]. This is frequently the case for many flight-capable insects that are captured unwittingly in traps provisioned with volatile chemicals intended to attract one or a few target species of insects [3, 4]. Substantial bycatch of a particular nontarget species may reveal a novel association between that species and the chemical [5–7]. Thus, this bycatch may be used to expand knowledge regarding the chemical ecology of various nontarget insects [4, 6].

Large bycatches of nontarget flies were sometimes unwittingly encountered in a study [8] that I conducted to evaluate responsiveness of arthropod natural enemies to various volatile chemicals among various field-crop habitats. Significant numbers of flies from three different families (Lauxaniidae, Chloropidae, and Anthomyiidae) were captured, and some captures represented potentially novel associations with volatile chemicals. The objective of this paper is to report on the associations of volatile chemicals with trap catch of the nontarget flies in my study.

2. Materials and Methods

Nontarget flies were captured unexpectedly on traps baited with individual volatile chemicals (Table 1; Figure 1) in seven separate field tests (Table 2) in 2004 and 2005 near Brookings, SD. As the catch of these flies was unexpected, their response to the volatile chemicals was evaluated on an ad hoc basis

Table 1: Volatile chemicals tested for attraction to beneficial insects in agricultural plots near Brookings, SD.

Volatile chemical		Alternate chemical name	Dosage, dispenser	
Year 2004				
Camphor	CAM	1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one	100 mg, cotton roll	Sigma-Aldrich, St. Louis, MO
Ethanol (nonattractant control)	ETH		100 mg, cotton roll	Sigma-Aldrich, St. Louis, MO
2-Isopropyl-3-methoxypyrazine	IMP	2-Isopropyl-3-methoxypyrazine	100 mg, cotton roll	Sigma-Aldrich, St. Louis, MO
2-Phenylethanol	PE	2-Phenylethanol	100 mg, cotton roll	Sigma-Aldrich, Milwaukee, WI
Terpineol (mixed isomers)	TERP	2-(4-Methyl-1-cyclohex-3-enyl)propan-2-ol	100 mg, cotton roll	Sigma-Aldrich, St. Louis, MO
trans-Caryophyllene	TC	*trans*-(1R,9S)-8-Methylene-4,11,11-trimethylbicyclo-undec-4-ene	100 mg, cotton roll	Spectrum Chemical, Gardena, CA
Year 2005				
4-Allylanisole	4AA	1-Methoxy-4-(2-propenyl)benzene	100 mg, cotton roll	Sigma-Aldrich, Milwaukee, WI
Ethanol (nonattractant control)	ETH		100 mg, cotton roll	Sigma-Aldrich, St. Louis, MO
Ethanol (nonattractant control)	ETCL		2 mg, commercial dispenser	ChemTica USA, Durant, OK
Eugenol	EUG	3-(3-Methoxy-4-hydroxyphenyl)prop-l-ene	100 mg, cotton roll	Sigma-Aldrich, Milwaukee, WI
Isoeugenol	ISO	3-(3-Methoxy-4-hydroxyphenyl)prop-2-ene	100 mg, cotton roll	Spectrum Chemical, Gardena, CA
Methyl salicylate	MS	Methyl 2-hydroxybenzoate	100 mg, cotton roll	Sigma-Aldrich, Milwaukee, WI
	MSCL		2 mg, commercial dispenser	ChemTica USA, Durant, OK
2-Phenylethanol	PE	2-Phenylethanol	100 mg, cotton roll	Sigma-Aldrich, Milwaukee, WI
	PECL		2 mg, commercial dispenser	ChemTica USA, Durant, OK

when meaningful numbers of the flies were caught in particular tests. The tests were conducted in 0.5 to 1.5-ha plots of spring wheat, corn, and soybean at the Eastern South Dakota Soil and Water Research Farm (44°19′N, 96°46′W, 500-m elevation). Crops in the plots were grown using common agronomic practices, with no insecticide applied to plots from the planting through the sampling periods each year.

Yellow sticky traps (Pherocon AM, Trecé, Adair, OK) were used for the tests. Each trap was folded along its midline so that two faces of adhesive surface were exposed for capturing insects. The traps were deployed individually on 1-m tall stakes and set just above the canopy (≈0.7-m ht.) in spring wheat and soybean plots and at the top of the stake in corn plots.

Traps were baited either with a cotton roll (3.8 cm long; Patterson, St. Paul, MN) impregnated with 100 mg of a volatile chemical or with a controlled-release, plastic sachet containing 2 mg of chemical (2-phenylethanol or methyl salicylate). A chemical was applied to a cotton roll by pipette as either stock solution or with the camphor treatment after dissolution in ethanol. Ethanol was used as the control. Wicks were prepared in the morning and clipped on a nonadhesive face of traps in the field a few hours later. The traps were deployed 10- to 30-m apart in the plots for various 2-day periods. Individual baited traps served as replicates, and treatments of volatile chemicals were replicated four to 10 times depending on the number tested and plot size.

The traps were retrieved from plots at the end of each test and taken to the laboratory and stored in refrigerators, and nontarget flies on them were identified and counted within a few days. The flies were identified to genus or species, depending on the condition of specimens on the traps; sex of the specimens was determined for Anthomyiidae by their dimorphic compound eyes (females, dichoptic; males, holoptic) [9]. Counts of nontarget flies for each 2-day trap period were subjected to separate analyses of variance by species and, for Anthomyiidae, by sex, using a generalized linear mixed model (PROC GLIMMIX [10, 11]). Treatment means were separated by the LSMEANS feature with Tukey-Kramer adjustment. Zero counts for the camphor treatment were omitted in order to support the robustness of parametric analyses in tests 1 and 2 [11].

Table 2: Tests of the attractancy of volatile chemicals to beneficial insects in agricultural plots near Brookings, SD.

Test	Crop	Date	Volatiles tested[1]	Flies evaluated
1	Corn	Aug 2–4, 2004	CAM, IMP, PE, TC, TERP, ETH	*Homoneura bispina* (Lauxaniidae); *Olcella* sp. (Chloropidae)
2	Soybean	Aug 17–19, 2004	CAM, IMP, PE, TC, TERP, ETH	*Olcella* sp.
3	Wheat	Jun 15–17, 2005	PE, PECL, MS, MSCL, ETH	Male *Delia* spp. (Anthomyiidae)
4	Corn	Jul 13–15, 2005	IMP, MS, PE, ETH	*H. bispina*, *Camptoprosopella borealis* (Lauxaniidae)
5	Corn	Jul 13–15, 2005	PECL, MSCL, ETCL	*H. bispina*, *C. borealis*
6	Soybean	Jul 14–16, 2005	PE, PECL, MS, MSCL, ETH	Male *Delia* spp.
7	Soybean	Jul 20–22, 2005	4AA, EUG, ISO, PE, ETH	Male and female *Delia* spp.

[1]See Table 1 for abbreviations.

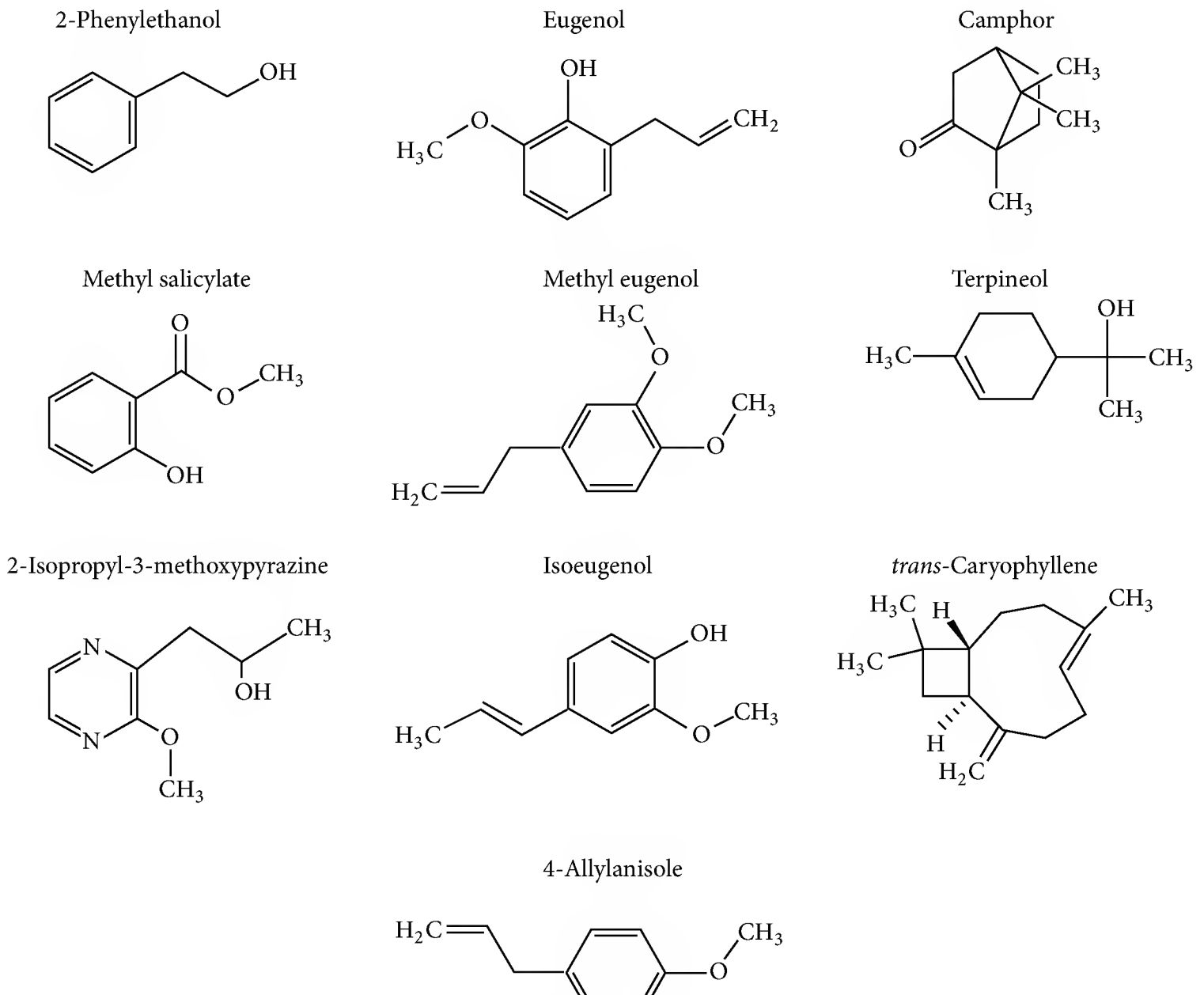


Figure 1: Chemical structures of test compounds.

3. Results

3.1. Overview. Significant numbers of flies from the families Lauxaniidae, Chloropidae, and Anthomyiidae were captured among the various tests. Trap counts of these flies often varied significantly among the volatile chemicals used to bait the traps. The results for each of these families are reported separately in the following.

3.2. Lauxaniidae. Trap catch of two species of lauxaniid flies, *Homoneura bispina* (Loew) and *Camptoprosopella borealis* Shewell, varied with volatile attractants in three tests in corn plots. In 2004 (test #1), trap catch of *H. bispina* varied with volatile attractant ($F = 94.63$; d.f. = 5, 53; and $P < 0.0001$). More *H. bispina* were caught on traps baited with 2-phenylethanol compared with those baited with any other compound (Figure 2). Trap catch did not differ among the other volatile compounds, except that terpineol-baited traps had fewer *H. bispina* than ones baited with ethanol.

A follow-up test (#4) in 2005 also showed that trap catch of *H. bispina* varied with volatile chemicals ($F = 13.31$; d.f. = 3, 36; and $P < 0.0001$). Results confirmed that 2-phenylethanol increased trap catch of *H. bispina* over other volatiles tested and showed that trap catches were higher on traps baited with methyl salicylate compared with those baited with ethanol or 2-isopropyl-3-methoxypyrazine (IMP) (Figure 3(a)).

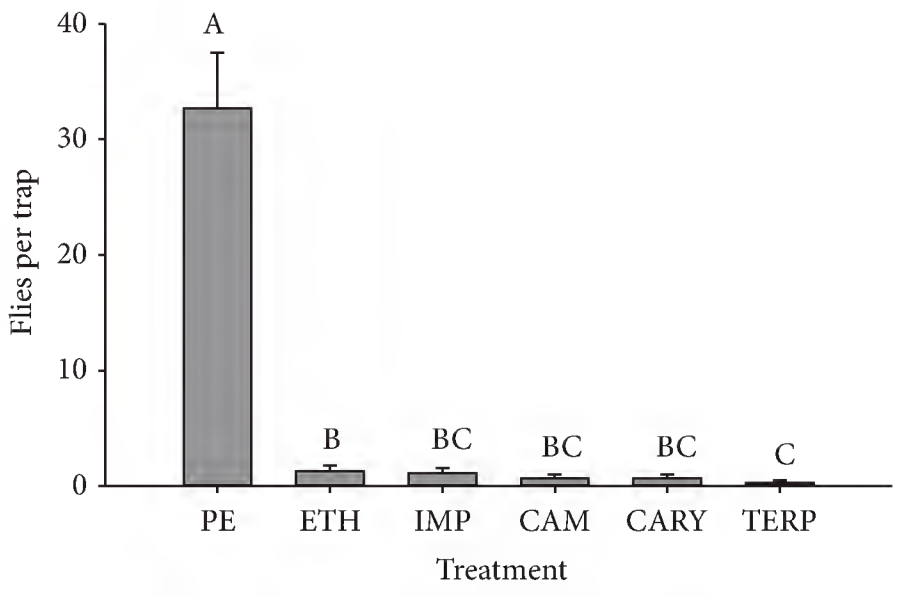


Figure 2: Mean number of adult *Homoneura bispina* per sticky trap (±SE) baited with volatile chemical from Aug 2 to Aug 4, 2004, in a corn plot near Brookings, SD (test #1). Bars without the same letter above have means that differ significantly. PE = 2-phenylethanol, ETH = ethanol (control), IMP = 2-isopropyl-3-methoxypyrazine, CAM = camphor, CARY = *trans*-caryophyllene, and TERP = terpineol, each at 100 mg on a cotton roll. *Homoneura bispina*, corn, 2004.

Catch of *H. bispina* did not differ between ethanol and IMP.

In addition, trap catch of *C. borealis* differed among volatiles in test #4 ($F = 5.20$; d.f. = 3, 36; and $P = 0.004$). Traps baited with methyl salicylate caught more *C. borealis* than those baited with ethanol (Figure 3(b)). Catch of *C. borealis* on traps baited with 2-phenylethanol or IMP did not differ from that on traps baited with either methyl salicylate or ethanol, and trap catch did not differ between 2-phenylethanol and IMP.

Trap catch of *H. bispina* and *C. borealis* each varied among volatiles in a final test using controlled-release lures (test #5). However, trap catch of lauxaniids was largely converse to that using cotton rolls as lures in test #4. That is, traps baited with methyl salicylate caught more *H. bispina* than those baited with 2-phenylethanol or ethanol (Figure 4(a); $F = 4.41$; d.f. = 2, 12; and $P = 0.037$), whereas traps baited with 2-phenylethanol caught more *C. borealis* than those baited with methyl salicylate or ethanol (Figure 4(b); $F = 5.16$; d.f. = 2, 12; and $P = 0.024$).

3.3. Chloropidae. Counts of an *Olcella* sp. varied among attractants in corn and soybean plots in 2004 (corn, test #1: $F = 188.66$; d.f. = 4, 44; and $P < 0.0001$; soybean, test #2: $F = 73.42$; d.f. = 4, 15; and $P < 0.0001$), as the counts were significantly more abundant on traps baited with IMP compared with traps baited with other volatile compounds. In corn, IMP-baited traps caught a mean (±SE) of 103.4 (±12.6) *Olcella* flies per trap, whereas counts averaged ≤3.3 *Olcella* flies per trap for each of the other treatments (Figure 5(a)). In soybean, IMP-baited traps caught a mean (±SE) of 83.8 (±9.1) *Olcella* flies per trap, whereas counts averaged ≤ 2.0 *Olcella* flies per trap for other treatments (Figure 5(b)). Traps baited with IMP and other attractants in 2005 (test #3) did not capture sufficient numbers of *Olcella* flies for analysis.

3.4. Anthomyiidae. Trap catch of adult *Delia* spp. varied by volatile chemicals in three tests. In spring wheat (test #3), catch of male *Delia* flies was greater on traps baited with 2-phenylethanol on a cotton wick than with all other treatments (Figure 6(a); $F = 27.47$; d.f. = 4, 25; and $P < 0.001$). Traps baited with 2-phenylethanol or methyl salicylate dispersed from a controlled-release dispenser captured more male *Delia* flies than traps baited with ethanol or methyl salicylate dispensed from cotton rolls. Numbers of female *Delia* flies did not vary among attractants ($P = 0.059$).

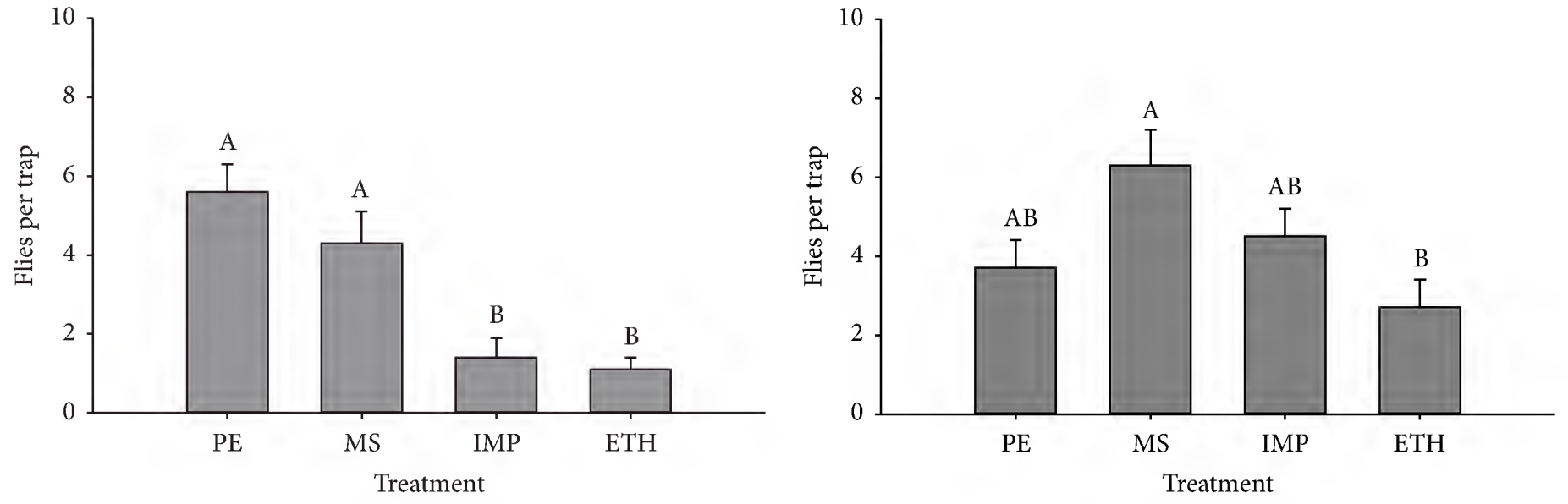
In soybean (test #6), the catch of male *Delia* flies was greater on traps baited with 2-phenylethanol on a cotton wick or in a controlled-release dispenser than on traps with other treatments (Figure 6(b); $F = 6.84$; d.f. = 4, 20; and $P = 0.001$). The numbers of male *Delia* flies did not differ among traps baited with a controlled-release lure of methyl salicylate or ones baited with cotton rolls imbued with ethanol or methyl salicylate. Trap catch of female *Delia* flies did not vary among volatile chemicals ($P = 0.16$).

In a second test in soybean (test #7), the trap catch of both male ($F = 17.25$; d.f. = 4, 20; and $P < 0.001$) and female *Delia* flies ($F = 4.20$; d.f. = 4, 20; and $P = 0.013$) varied among volatile chemicals. More male and female *Delia* flies were captured on traps baited with 2-phenylethanol than on those baited with other volatile chemicals (Figure 7); trap catch of *Delia* spp. did not vary among eugenol, isoeugenol, 4-allylanisole, or ethanol.

4. Discussion

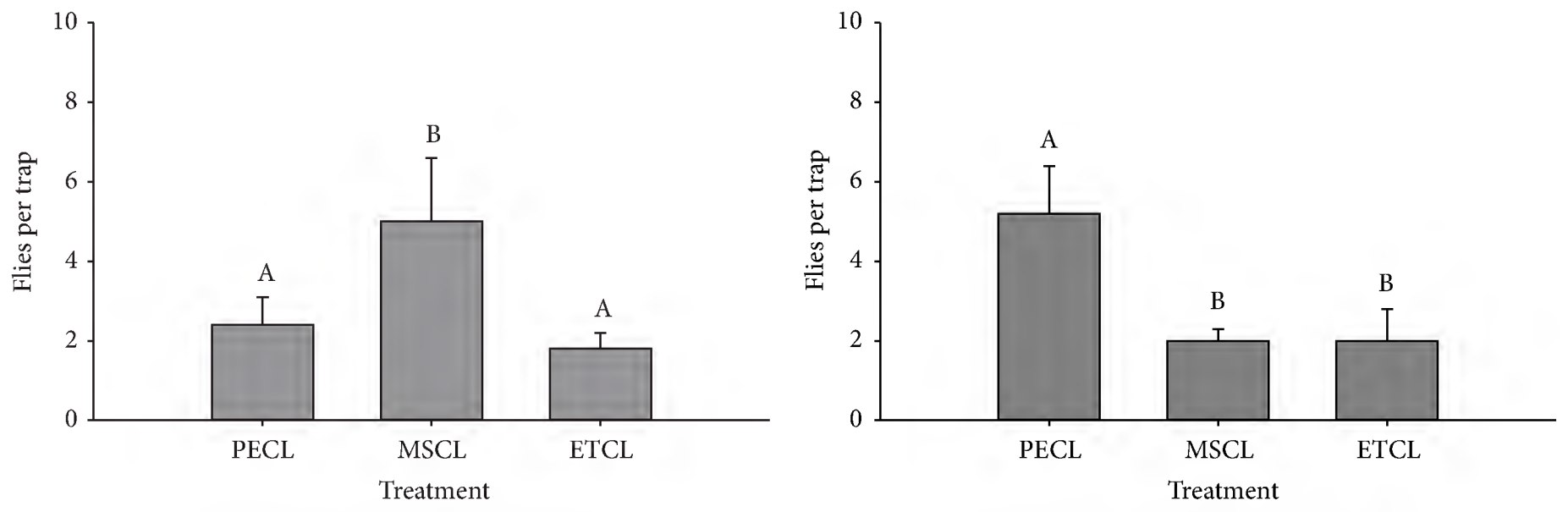
Some of the findings in this study represent novel reports of attractancy for particular volatile compounds to the two lauxaniid species, *Homoneura bispina* and *Camptoprosopella borealis*, and to the chloropid flies in the genus *Olcella*. However, previous reports have documented responses of *Delia* flies to traps baited with volatile attractants such as 2-phenylethanol [12]. Results of this study are compared below with findings of other studies for each group of flies.

4.1. Lauxaniidae. The attractancy of 2-phenylethanol and methyl salicylate to lauxaniids is novel, but the basis for their attraction is unclear. Consideration of lauxaniid biology and the natural occurrence of 2-phenylethanol and methyl salicylate may allow for inference about the basis of attraction for these two volatiles. Specific knowledge about the biology of *H. bispina* and *C. borealis* is lacking. Adult lauxaniids generally feed on fungi [13] and visit flowers [14], and larvae are typically saprophagous among fallen leaves, straw, rotting wood, and bird nests [15]. With regard to the chemicals, 2-phenylethanol is a compound associated with decaying vegetation [16], and methyl salicylate is induced in plants by herbivory to act as a volatile attractant of herbivore natural enemies [17]. However, 2-phenylethanol and methyl salicylate are also floral volatiles that attract pollinators [17, 18]. Thus, given the biology of lauxaniids, the increased trap catch of *H. bispina* and *C. borealis* to 2-phenylethanol and methyl salicylate may reflect an attraction to these compounds as floral attractants or as decaying vegetation that



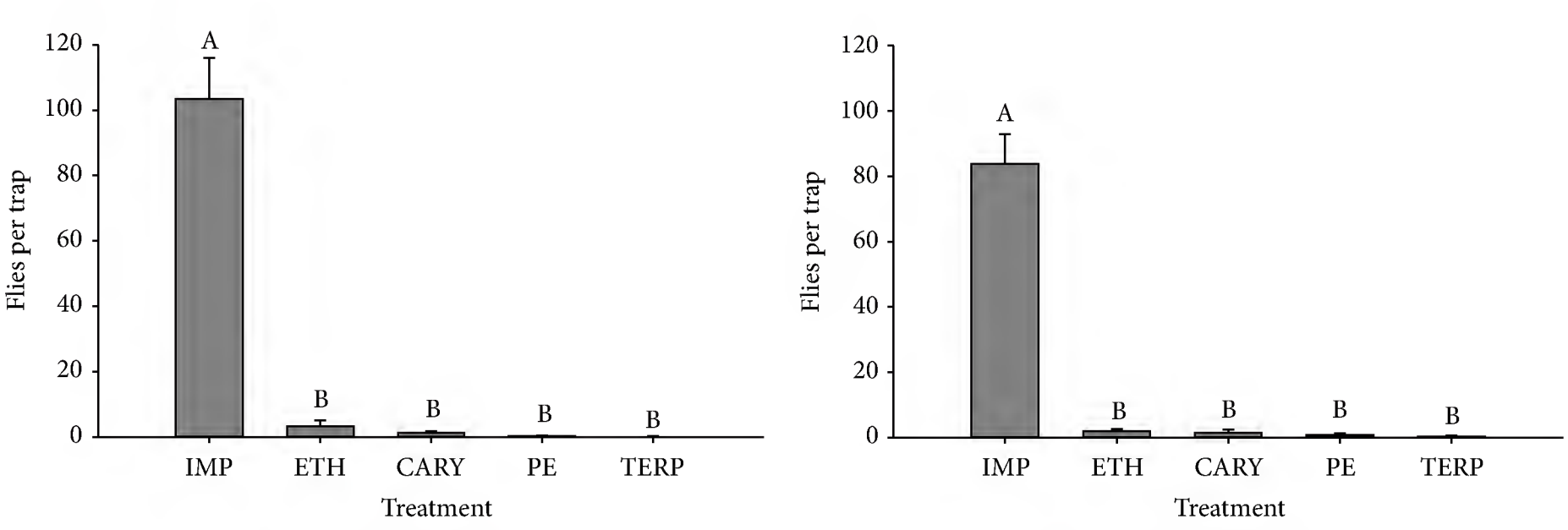
(a) *Homoneura bispina*, corn, 2005 (b) *Camptoprosopella borealis*, corn, 2005

FIGURE 3: Mean number of lauxaniid flies per sticky trap (±SE) baited with volatile chemicals from Jul 13 to Jul 15, 2005, in a corn plot near Brookings, SD (test #4). For each species, bars without the same letters above them indicate that the means differ significantly. PE = 2-phenylethanol, MS = methyl salicylate, IMP = 2-isopropyl-3-methoxypyrazine, and ETH = ethanol (control), each at 100 mg on a cotton roll. (a) Adult *Homoneura bispina*; (b) adult *Camptoprosopella borealis*.



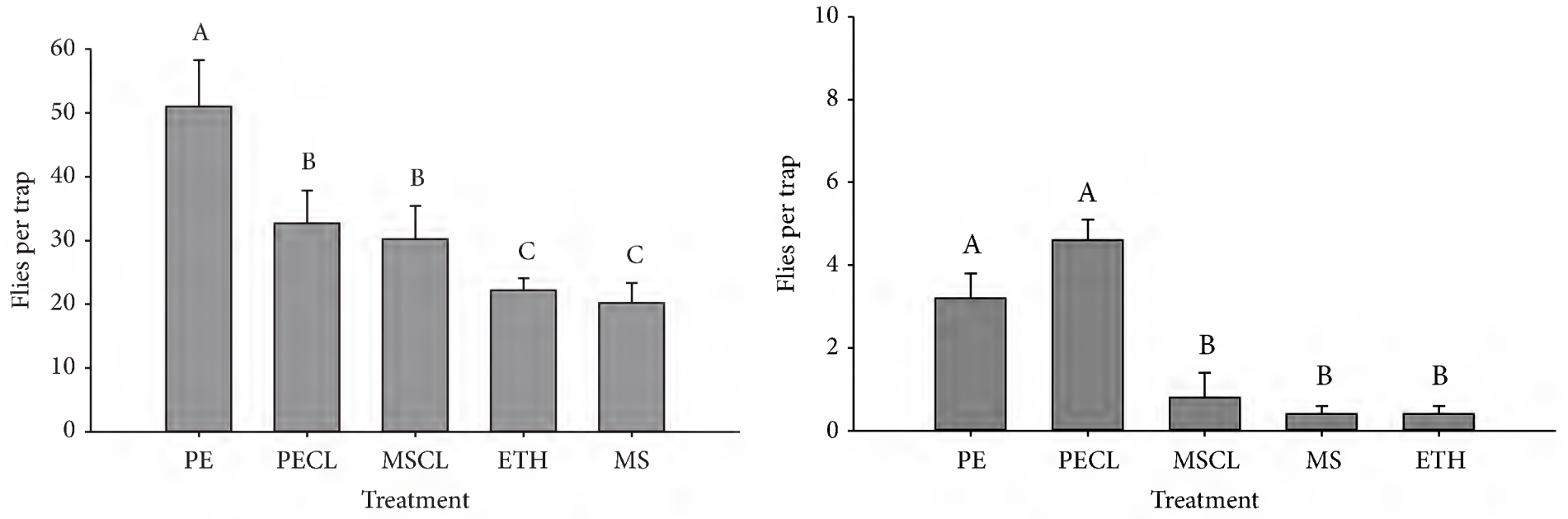
(a) *Homoneura bispina*, corn, 2005 (b) *Camptoprosopella borealis*, corn, 2005

FIGURE 4: Number of lauxaniid flies per sticky trap (±SE) baited with 2 mg in controlled-release dispensers of ethanol (control) = ETCL, methyl salicylate = MSCL, or 2-phenylethanol = PECL in corn plots near Brookings, SD, Jul 13–15, 2005 (test #5). (a) Adult *Homoneura bispina*; (b) adult *Camptoprosopella borealis*. For each species, bars without the same letter above them indicate that the means differ significantly.



(a) *Olcella* flies, corn, 2004 (b) *Olcella* flies, soybean, 2004

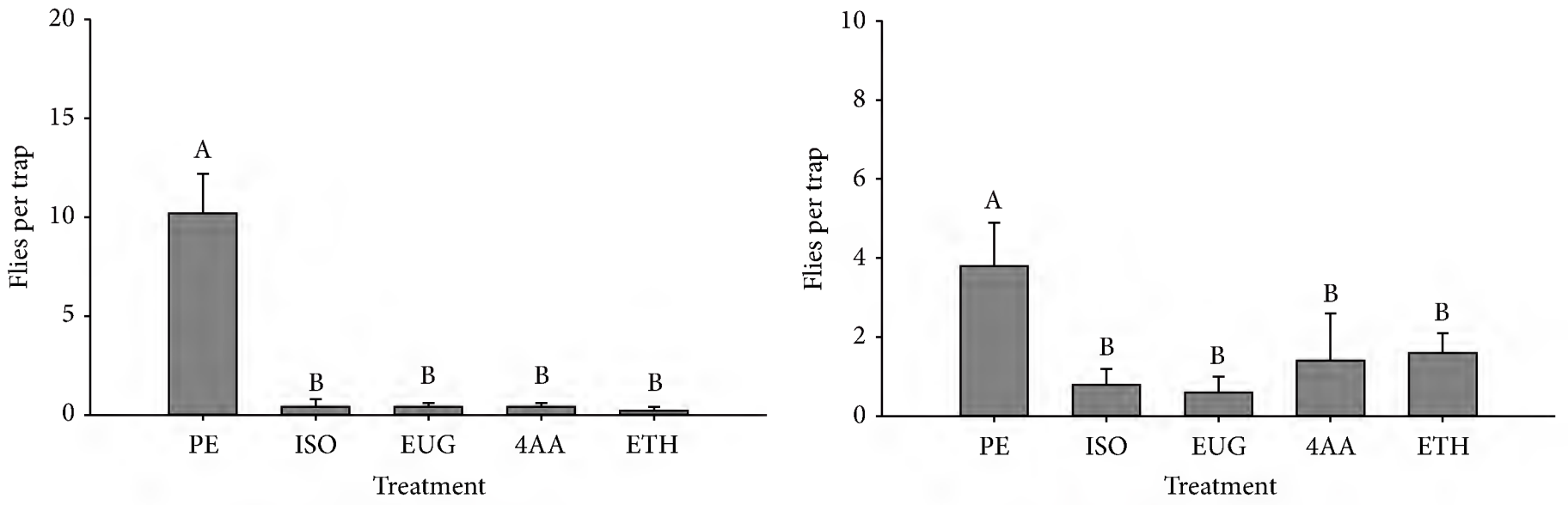
FIGURE 5: Mean number of adult *Olcella* sp. flies per sticky trap (±SE) baited with 100 mg of volatile chemicals in plots near Brookings, SD. Chemicals dispensed on cotton rolls. Bars without the same letters above them indicate that the means differ significantly. (a) Aug 2–4, 2004, corn plot (test #1). (b) Aug 17–19, 2004, soybean (test #2). IMP = 2-isopropyl-3-methoxypyrazine, ETH = ethanol (control), CARY = *trans*-caryophyllene, PE = 2-phenylethanol, and TERP = terpineol. Zero counts for camphor (CAM) traps not included.



(a) ♂♂ *Delia* flies, spring wheat, 2005

(b) ♂♂ *Delia* flies, soybean, 2005

FIGURE 6: Mean number (±SE) of male *Delia* flies captured near Brookings, SD, on sticky traps baited with a volatile attractant. (a) Spring wheat, Jun 15–17, 2005 (test #3). (b) soybean, Jul 14–16, 2005 (test #6). PE = 2-phenylethanol, MS = methyl salicylate, and ETH = ethanol (control), each at 100 mg on a cotton roll; PECL = 2-phenylethanol and MSCL = methyl salicylate, each at 2 mg on a controlled-release lure. For each graph, bars without the same letter above them indicate significant differences between means.



(a) ♂♂ *Delia* flies, soybean, 2005

(b) ♀♀ *Delia* flies, soybean, 2005

FIGURE 7: Mean number (±SE) of male (a) and female (b) *Delia* flies captured on sticky traps baited with volatile chemical, Jul 20–22, 2005, in a soybean plot near Brookings, SD (test #7). For each sex, bars without the same letters above them indicate that the means differ significantly. PE = 2-phenylethanol, EUG = eugenol; ISO = isoeugenol; 4AA = 4-allylanisole, and ETH = ethanol (control), each at 100 mg on a cotton roll.

may indicate suitable larval habitat to ovipositing females. The differential responses of the two species to these compounds emanating from either cotton rolls or controlled-release septa indicate that concentration and release rate influence lauxaniid response. Both cotton and plastic have been used effectively in studies with volatile attractants and flies; however, dispenser type may be a factor affecting trap catch, as different dispensers release volatile compounds at different rates [19, 20]. Further study is needed to clarify these relationships.

4.2. Olcella Flies. The results of my study constitute the first report of attractancy of 2-isopropyl-3-methoxypyrazine (IMP) to *Olcella* sp. (and other insects). It significantly attracted large numbers of *Olcella* sp. in two tests in August 2004 but failed to catch meaningful numbers of *Olcella* sp. in a July 2005 test. The phenology of *Olcella* flies is not known for field crops in South Dakota. However, as sampling of insects directly relates to their abundance and activity, and trap catch can reflect the temporal dynamics of insects [21, 22], I hypothesize that the disparity in trap catch between years may have been due to differences in abundance or activity of *Olcella* flies at the respective times of year (July versus August) when the tests were run. I suggest future tests across a broader time frame to determine the temporal dynamics involved in attraction of *Olcella* flies to IMP.

The basis of attractancy of IMP to *Olcella* sp. is unclear. IMP is a pungent volatile and structural analog of methoxyalkylpyrazines (MAP), which are defensive compounds of aposematic insects [23, 24]. As such, IMP was originally intended as a repellent control in screening volatile compounds for natural enemy responsiveness, when its unexpected attractancy to *Olcella* was discovered. Other studies have shown that *Olcella* spp. are attracted to various volatile compounds [6, 25–27]. For instance, *Olcella trigramma* (Loew) is attracted to hexyl butyrate and (*E*)-2-hexenyl

butyrate, defensive compounds in the metathoracic scent glands of stink bugs (Hemiptera: Pentatomidae) [27] that seep out upon death [28]. Furthermore, chloropid flies are attracted to stink bugs and other true bugs (Hemiptera) that emit defensive secretions when being trapped in spider webs [29], and Zhang and Aldrich [27] have suggested that these defensive compounds function as kairomones to scavenging flies. Accordingly, I hypothesize that the increased trap catch of *Olcella* flies in my study was due to analogous attraction of these flies to IMP as a defensive secretion and suggest further testing of IMP and other MAPs for their attractancy to chloropids.

Alternatively, however, if *Olcella* flies are attracted to defensive compounds of trapped insects (e.g., in spider webs [27]), their attraction to IMP-baited traps could have ultimately been influenced by the capture of other insects on those traps. However, capture of other insects was not appreciable on IMP-baited traps in the present study. Thus, the capture of high numbers of *Olcella* flies was likely due to their direct attraction to IMP.

4.3. Delia Flies. Previous reports have shown attractancy of 2-phenylethanol to *Delia* spp., and male *Delia* are particularly responsive to this compound [12, 16, 30]. 2-Phenylethanol was shown to be a key component of decomposing onion pulp that attracted onion flies [*D. (=Hylemya) antiqua* (Meigen)] and seedcorn maggot flies [*D. (=Hylemya) platura* (Meigen)]. Correspondingly, attraction of *Delia* spp. to 2-phenylethanol has been documented in onion [12, 16, 30] but also in snap bean fields [12]. However, my study is the first known to document a trapping response of *Delia* spp. to 2-phenylethanol in wheat and soybean fields. Some species, such as *D. platura* and *D. florilega* (Zetterstedt) (bean seed maggot), are pests of seedling soybean [31], especially in fields where ovipositing females are attracted to soil that has had manure applied or green plant material incorporated [32]. Traps baited with attractants such as 2-phenylethanol might be useful as a monitoring tool to assess *Delia* populations in soybean fields [12]. However, tests may be needed to determine how the presence of competing volatiles associated with manure and decaying green plant material might affect the catch of traps baited with synthetic volatile attractants.

5. Conclusions

In summary, three types of flies were caught serendipitously in this study on yellow sticky traps that had been baited with a relatively limited range of attractants and placed in a limited number of field-crop habitats at a single location in eastern South Dakota. As such, there is a rich set of follow-up questions that may be pursued systematically in future studies at various geographic locations. These studies may include tests of chemical analogs and varied dosages of the volatiles used here in order to determine structure-activity relationships and sensitivity involved in the flies' attraction. Future studies may be conducted to determine if response varies by sex and reproductive status, particularly for lauxaniids and *Olcella* flies. In addition, future studies may also test attractancy among dispensers with different release rates [19] and across different trap designs, including traps ones that leave flies intact upon removal and thereby facilitate researchers' ability to readily identify flies to species. Finally, additional studies on the basic biology of *H. bispina*, *C. borealis*, and *Olcella* flies are needed to make inferences about the bases for responses to volatile chemicals. The suggested studies are likely to greatly expand knowledge about the chemical ecology of these groups of flies.

Disclosure

The paper reports research results only. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by USDA and does not imply its approval to the exclusion of other products that may also be suitable.

Competing Interests

The author declares that there are no competing interests regarding the publication of this paper.

Authors' Contributions

Eric Beckendorf, David Mills, Kendra Jensen, Hanna Fetzer, Joshua Pedro, and Ryan Rubbelke provided technical assistance. Stephen Gaimari and Jon Kieckhefer identified flies. Mark West advised on statistical analysis. Sharon Papiernik and Fathi Halaweish provided advice on drawing chemical structures. Jan Menely, Michael Catangui, and Leslie Hammack advised on attractants. Eric Beckendorf, Lauren Hesler, Deirdre Prischmann-Voldseth, Leslie Hammack, and Sharon Papiernik graciously reviewed drafts of this paper.

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Research Article

Ovarian Development and Vitellogenin Gene Expression under Heat Stress in Silkworm, *Bombyx mori*

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The present study observed the effect of heat stress on ovarian development, fecundity, and vitellogenin gene expression in silkworm, *Bombyx mori*. The result showed that the heat shock treatment to spinning larvae and pupae at 39°C (1 h and 2 h) did not cause any adverse effect on the reproductive performance of *B. mori*. However, the heat shock treatment at 42°C or above caused a decrease in the fecundity. The heat shock treatment to day 2 pupae for 2 h at 45°C caused a drastic effect on the development of ovary as measured by gonadosomatic index. The study thus showed that a brief exposure of *Bombyx* larvae and pupae to a temperature of 42°C or higher, much prevalent in tropical countries like India, greatly affects the ovarian development and reproductive performance of this commercially important insect. The study further showed a developmental- and tissue-specific expression of vitellogenin mRNA in fat body and ovary upon heat shock. When heat shock treatment was done at 39°C and 42°C to spinning larvae, ovary showed an upregulation in the expression of vitellogenin mRNA, whereas fat body failed to do so. However, at 45°C, both fat body and ovary showed a downregulation. The heat shock treatment to day 2 pupae showed an upregulation in the vitellogenin mRNA expression in both fat body and ovary, even at 45°C. The upregulation in the expression of vitellogenin upon heat shock indicates its role in thermal protection of *Bombyx* larvae and pupae.

1. Introduction

Global warming is one of the major challenges for the survival and reproduction of many life forms including insects. The increase of global mean surface temperature is likely to be 0.3–4.8°C by the end of 21st century relative to 1986–2005 [1]. It is warned that the heat waves will occur with a higher frequency and a longer duration [1]. Studies have shown that warmer temperatures associated with climate change can potentially affect insect species' population dynamics directly through effects on survival, generation time, fecundity, and dispersal [2, 3]. Insects, being ectothermic organisms, are very likely to respond quickly to increased temperatures [4]. However the response of individual insect species will depend on their geographical range, trophic level, and natural history [3]. For majority of temperate insect species, increased temperature will allow them to extend their geographical range and enhance their population fitness [2]. However, for tropical insects, increased temperature is likely to have the most deleterious consequences as tropical insects are relatively sensitive to temperature change and are currently living very close to their optimal temperature [5]. Temperature above the optimum temperature is perceived as heat stress by all living organisms.

In a tropical country like India environmental factors such as temperature and humidity play a major role in the success of sericulture industry. Because of the extensive and careful domestication over centuries, the silkworm, *Bombyx mori,* is very much susceptible to abrupt temperature changes. Studies have shown that cells of all known organisms including *B. mori* respond to stress such as temperature by increased synthesis of heat shock or stress proteins (Hsps) [6–10]. Accumulation of these stress proteins induced by exposure to mild stress results in a transient state of stress resistance. It is indicated that heat inducible Hsps are important for heat induced hormesis in longevity and heat stress resistance in *Drosophila* [11]. Hormesis is the phenomenon where a mild exposure to an otherwise detrimental factor such as

heat, insecticides, and radiation becomes beneficial for many organism and life history traits [12–15]. In *Drosophila*, a significant increase in lifespan of females, repeatedly exposed to mild heat stress through hormesis, has been suggested by Hercus et al. [16]. In the oriental fruit moth, *Grapholita molesta*, the exposure of females to a single heat event at 38°C for 4 h caused an increase in the lifespan and length of the oviposition period, leading to a potential increase in lifetime fecundity and suggesting hormesis [17]. However, Forbes [18] suggested that not all aspects of organism performance can be hormetic simultaneously and there might be an energetic trade-off among life history traits. Since both survival and reproduction require energy and since energy is limited, it is expected that there will be a trade-off between these two fitness components [19].

In *B. mori,* although literatures are available on the effect of heat stress on survival and expression of heat shock proteins [8, 20–25], little information is available on the effect of heat stress on its reproductive fitness. It is suggested that though the increased expression of heat shock proteins under mild heat stress plays an important role in helping insect to survive, its high level usually brings negative physiological effects on organisms [26]. The improved thermotolerance at the cost of reduced reproductive performance has been observed in many insect species such as *Bicyclus anynana* and *Helicoverpa armigera* [27–30]. In *Spodoptera litura* and *Neoseiulus barkeri*, mild thermal stress increases thermotolerance but brings a detrimental effect on fertility and fecundity [26, 31].

The developing ovary in *Bombyx* consists of four polytrophic meroistic ovarioles, each of which contains a long linear array of developing follicles in progressively advancing stage of development. Each follicle is surrounded by a layer of follicle cells and contains an oocyte and interconnected nurse cells. Vitellogenin, a precursor of major egg yolk protein, plays an important role in promoting the growth and differentiation of developing oocytes. The regulation of vitellogenin is directly under the control of hormones at the transcriptional level [32]. In *Bombyx*, its biosynthesis is regulated transcriptionally in a sex- and stage-dependent manner [33]. Vitellogenin functions not only as an energy reserve for the developing embryo but also in innate immune response [34]. Singh et al. [35] showed the antibacterial activity of vitellogenin and found that the infected silkworm larvae, treated with purified vitellogenin, survived the full life cycle in contrast to untreated animals. Increased vitellogenin expression in *Caenorhabditis elegans* potently increases their resistance against pathogenic bacteria and heat [36]. Recently Ihle et al. [37] showed that aging and lifespan in honey bees are affected by vitellogenin. This protein influences worker lifespan both as a regulator of behavioral maturation and through antioxidant and immune functions as an experimental reduction of vitellogenin expression resulted in decreased lifespan and increased susceptibility to oxidative damage.

Since development time and reproductive output of an insect are nearly as important to population growth as the survival of individuals, the present study has been carried out to investigate the consequences of heat stress on ovarian development and fecundity as well as on vitellogenin gene expression in *B. mori*.

2. Material and Methods

2.1. Insect. Silkworm (*Bombyx mori*) L. (CSR 18, a bivoltine race) was used for present study. Disease-free layings of silkworms breed CSR 18 were obtained from Central Sericultural Germplasm Resource Centre, CSB, Hosur, Tamil Nadu, and reared with fresh mulberry leaves in laboratory under controlled conditions (temperature, 25±1°C; humidity, 60–90%, photoperiod, 12L : 12D). Larvae and pupae were staged according to days after ecdysis and the available morphological markers such as spinneret pigmentation and gut purge.

2.2. Heat Shock Treatment. For heat shock treatment, day 3 spinning larvae and day 2 pupae were placed individually in glass test tubes plugged with cotton and were submerged in a water bath at 39°C, 42°C, and 45°C for 1 h and 2 h, respectively, as described [38]. After the treatment, the larvae and pupae were returned to controlled laboratory conditions. Female larvae and pupae were considered for heat shock treatment. Male silkworm larvae and pupae were subjected to heat shock treatment only for the purpose of copulation.

2.3. Determination of Gonadosomatic Index. Heat shock was given to day 2 female pupae and developing ovaries were dissected out after 48 h (day 4), 96 h (day 6), and 144 h (day 8) of heat shock treatment. The isolated ovaries were rinsed with insect ringer solution, blotted dry with filter paper, and the weight was measured using digital weighing balance. Gonadosomatic index (GSI) was calculated using the equation GSI = ovary wet weight (g)/total body weight (g) × 100 [39]. The experiments were repeated 2-3 times and mean value of GSI was calculated for each experimental group from the data obtained from 6–8 individuals.

2.4. Estimation of Fecundity. Day 3 spinning larvae and day 2 pupae (both males and females) were subjected to heat shock treatment and the adult moths were collected for mating. The moths were allowed to copulate for 5 h and afterwards the moths were decoupled and the females were kept for oviposition and males were disposed off. The fecundity was calculated by counting the number of eggs laid by each female within 24 h time period. The experiments were repeated 3 times and the mean value of fecundity was calculated from the data obtained from 10–12 individuals.

2.5. RNA Isolation and Semiquantitative RT-PCR. The fat body and ovary were dissected out from 5th instar larvae and pupae under cold insect ringer solution and homogenized immediately in TRIzol reagent (Invitrogen) and stored at −70°C. Total RNA was isolated according to the manufacturer's protocol. The concentration of RNA was measured spectrophotometrically at 260 nm. The purity of the total RNA was assessed using $A_{260/280}$ ratios. Denaturing agarose

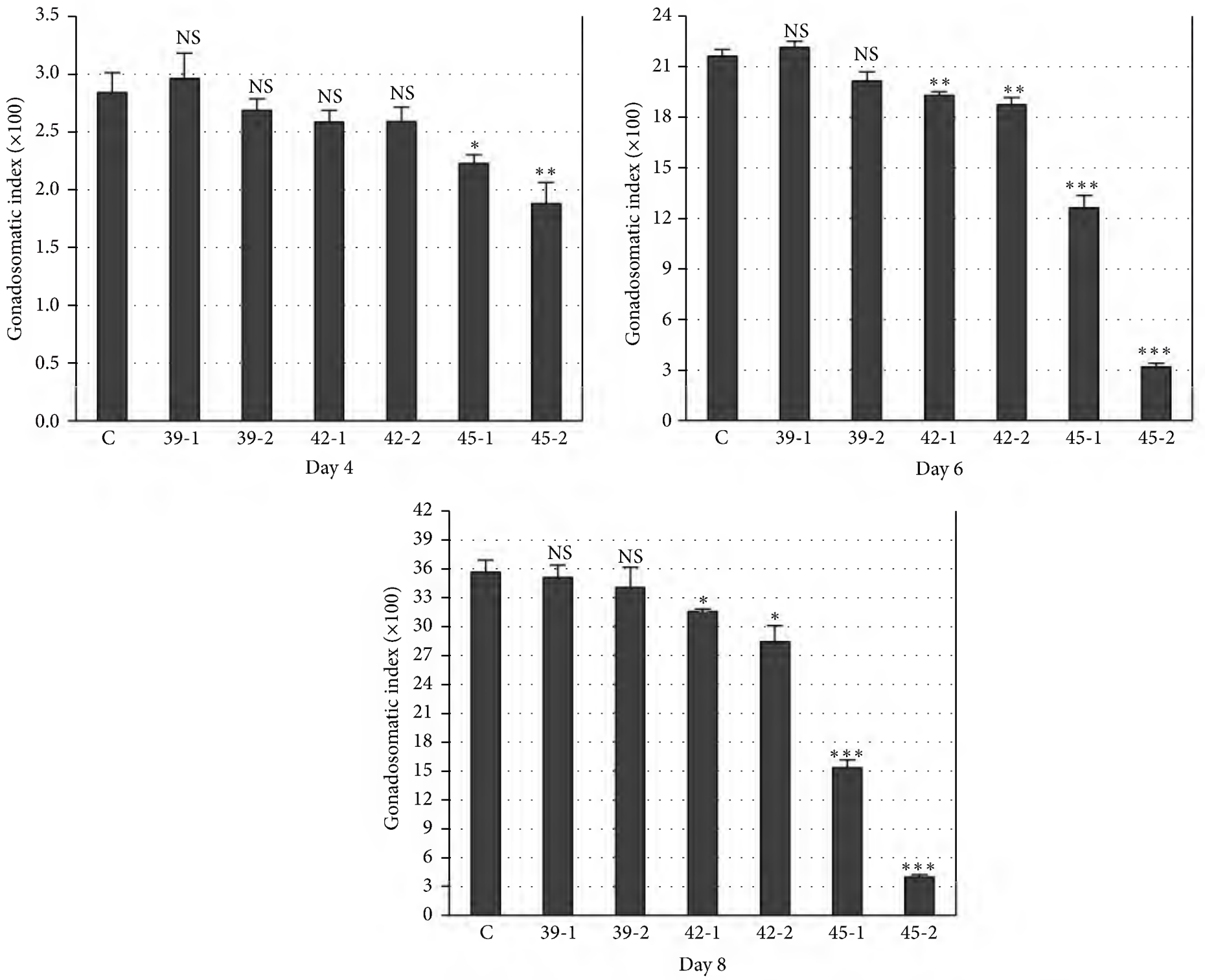


FIGURE 1: Effect of heat stress on gonadosomatic index (ovary). Heat shock was given to pupae, 2 days after larval-pupal ecdysis and developing ovarioles were dissected out from pupae on days 4, 6, and 8 from each experimental group. The wet weight of developing ovaries was measured after rinsing with insect ringer solution and gonadosomatic index was calculated with respect to total body weight. Each histogram bar represents the mean gonadosomatic index ($n = 6$–8) and the error bar represents standard error of the mean (SEM). The asterisks show the data which are significantly different from control sample (unpaired t-test; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). NS: not significant at $p < 0.05$. C: control; 39-1: heat shock at 39°C for 1 h; 39-2: heat shock at 39°C for 2 h; 42-1: heat shock at 42°C for 1 h; 42-2: heat shock at 42°C for 2 h; 45-1: heat shock at 45°C for 1 h; 45-2: heat shock at 45°C for 2 h.

gel electrophoresis was performed to ascertain the integrity of isolated RNA.

The cDNA was synthesized from total RNA using M-MLV reverse transcriptase (Invitrogen) and oligo dT 18 primers (Integrated DNA Technologies, Inc.) following manufacturer's protocol. The cDNA was subsequently used for amplification of target DNA by Polymerase Chain Reaction (PCR) using the gene specific forward and reverse primers. Forward and reverse primers for vitellogenin and ribosomal protein (rp49) were designed based on the sequence data available on Genbank. For vitellogenin (Accession number: NM_001043844) the forward primer and reverse primers are 5′-GCTTTGCCTAGGACCCTACG-3′ and 5′-GCAGCGGACTTAAAAGCAACC-3′ and for rp49 (Accession number: AB048205) the forward primer and reverse primers are 5′-GCATCAATCGGATCGCTATGAC-3′ and 5′-CAAGAAGACCCGTCATATGCT-3′. Briefly, the samples were heated at 95°C for 10 min followed by 30 amplification cycles (95°C for 30 s, 60°C for 30 s, and 72°C for 45 s) and a final 10 minutes extension period at 72°C. The amplified products were subjected to 3.5% agarose gel electrophoresis and molecular size was confirmed by running DNA standard. Images of the RT-PCR ethidium bromide-stained gel were acquired and quantification of the band densities was performed using Image-J program. The experiments were repeated 2-3 times with 2-3 independent biological replicates in each experiment. For data normalization, *rp49* was used as a reference gene. The transcript level of the vitellogenin gene was calculated relative to *rp49* expression for each sample by comparing the band densities. The normalized value of vitellogenin mRNA expression level in one of the control samples was designated as 1 and afterwards the relative

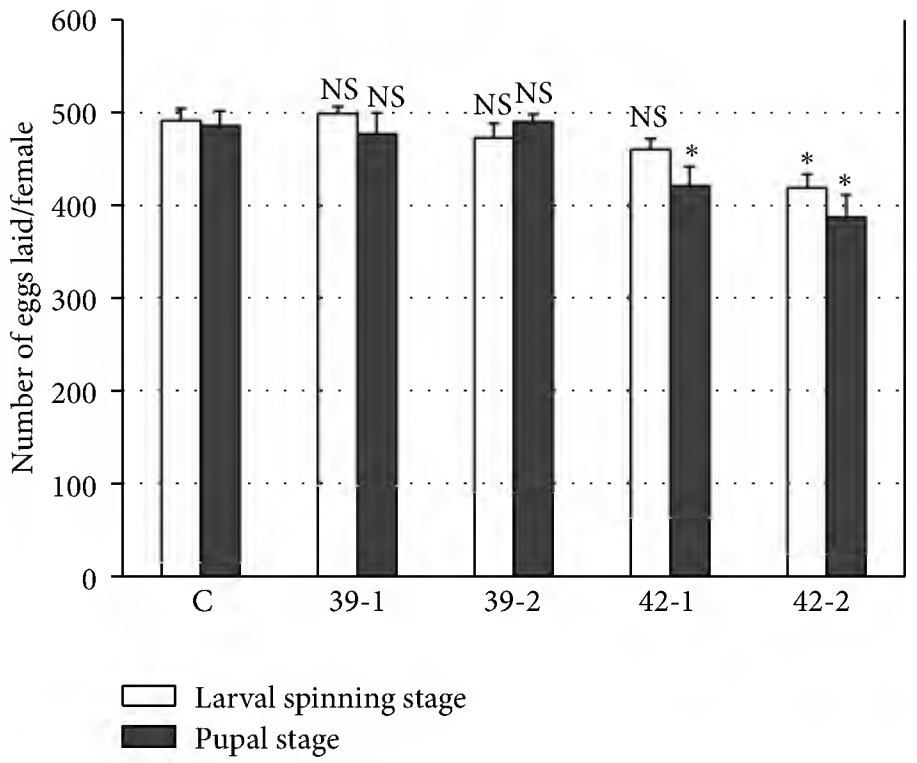


FIGURE 2: Effect of heat stress on fecundity. Heat shock was given to spinning larvae and day 2 pupae and number of eggs laid per female was counted from each experimental group. Each histogram bar represents the mean value for fecundity (n = 10–12) and the error bar represents standard error of the mean (SEM). The asterisks show the data which are significantly different from control sample (unpaired t-test; $^{*}p < 0.01$). The abbreviations used are as mentioned for Figure 1.

expression levels for the remaining control samples and the experimental samples were calculated. Mean and SEM were obtained using relative expression level values for control and all experimental groups.

2.6. Statistical Analysis. Student's t-test was performed for the analysis of the data using GraphPad Prism 6 software.

3. Results

3.1. Effect of Heat Stress on Ovarian Development and GSI. Gonadosomatic index is an important indicator to show the gonadal developmental progress and its maturity. In the present study, *Bombyx* ovary showed an increase in GSI with the advancement of ovarian development during pupal stage. The GSI increased from 2.8 ± 0.2 to 21.6 ± 0.4 from day 4 to day 6 of pupal life (Figure 1). On day 8, GSI increased to 35.6 ± 1.3. The mild heat shock treatment to day 2 pupae at 39°C (1 h and 2 h) failed to show any adverse effect on GSI. However, the heat shock treatment at 42°C and 45°C caused a severe effect on GSI. The heat shock exposure at 42°C (1 h and 2 h) caused a significant decrease ($p < 0.05$–0.01) in GSI, as observed on day 6 and day 8 of pupal life (Figure 1). The heat shock treatment at 45°C showed a more severe effect than 42°C and even day 4 pupae showed a decrease in GSI (Figure 1). With 1 h exposure at 45°C, a decrease in GSI to 12.6 ± 0.7 and 15.3 ± 0.8 was observed on day 6 and day 8, respectively (Figure 1). With the increase of heat shock duration to 2 h at 45°C, a drastic decrease in GSI to 3.2 ± 0.23 and 4.0 ± 0.2 was observed on day 6 and day 8 of pupal life, respectively (Figure 1).

3.2. Effect of Heat Stress on Fecundity. The heat shock treatment to either spinning larvae or day 2 pupae at 39°C failed to bring any adverse effect on the fecundity of the adult female moth (Figure 2). However, the heat shock treatment at 42°C caused a significant decrease in fecundity. The heat shock exposure to day 2 pupae for 1 h at 42°C caused a decrease in fecundity to 421 ± 20 from 491 ± 12, observed for untreated adult moth (Figure 2). A 2 h exposure at this temperature showed a further decrease in fecundity to 387 ± 24. For spinning larvae, the heat shock treatment at 42°C for 1 h did not show any significant changes in the fecundity of the survived adult. However, the exposure to spinning larvae for 2 h at this temperature caused a significant decrease in fecundity to 418 ± 14 (Figure 2). The fecundity data could not be measured for experimental group, heat stressed at 45°C, as this treatment caused death of the most of the heat stressed individual, although the heat stressed pupae could survive for few days, as evident by a response to a stimulus like touch [25].

3.3. Effect of Heat Stress on Vitellogenin mRNA Expression. The heat shock treatment to spinning larvae and pupae showed a developmental- and tissue-specific expression of vitellogenin mRNA in fat body and ovary. The heat shock treatment to spinning larvae at mild and mild-to-severe heat stressed condition (39°C and 42°C) showed a significant increase in vitellogenin mRNA expression level but only in ovary (Figure 3(b)) and not in fat body (Figure 3(a)). However, at severe-to-lethal heat stressed condition (45°C), ovary showed a significant decrease in vitellogenin expression (Figure 3(b)). The fat body tissues also showed a decrease in vitellogenin expression at this temperature but only for a 2 h exposure (Figure 3(a)).

The heat shock treatment to day 2 pupae showed an unexpected but a very promising result on vitellogenin mRNA expression level. In both fat body and ovary, an increase in vitellogenin mRNA expression was observed upon heat shock treatment even at severe-to-lethal heat stressed condition (45°C) (Figures 4(a) and 4(b)). It should be noted that the heat stressed pupae at 45°C could survive for many days after the heat shock treatment, although they could not emerge as adults. In day 4 pupae, however, vitellogenin expression in both fat body and ovary tissues decreased to almost control level, except at 45°C, 2 h, where a downregulation in vitellogenin expression was observed (Figure 4(c)).

4. Discussion

Our previous study [25] showed an increased expression of heat shock protein 90 (hsp90) in *Bombyx* larvae and pupae upon mild heat shock treatment at 39°C. The study further suggested that the upregulation of hsp90 at a particular heat shock temperature is associated with the ability of the animal to survive upon heat stress. The present study showed that heat shock to Bombyx larvae and pupae at mild temperature (39°C) is without any adverse effect on ovarian development and fecundity. Thus, the ability of silkworm larvae and pupae to withstand heat stress at mild temperature apparently with

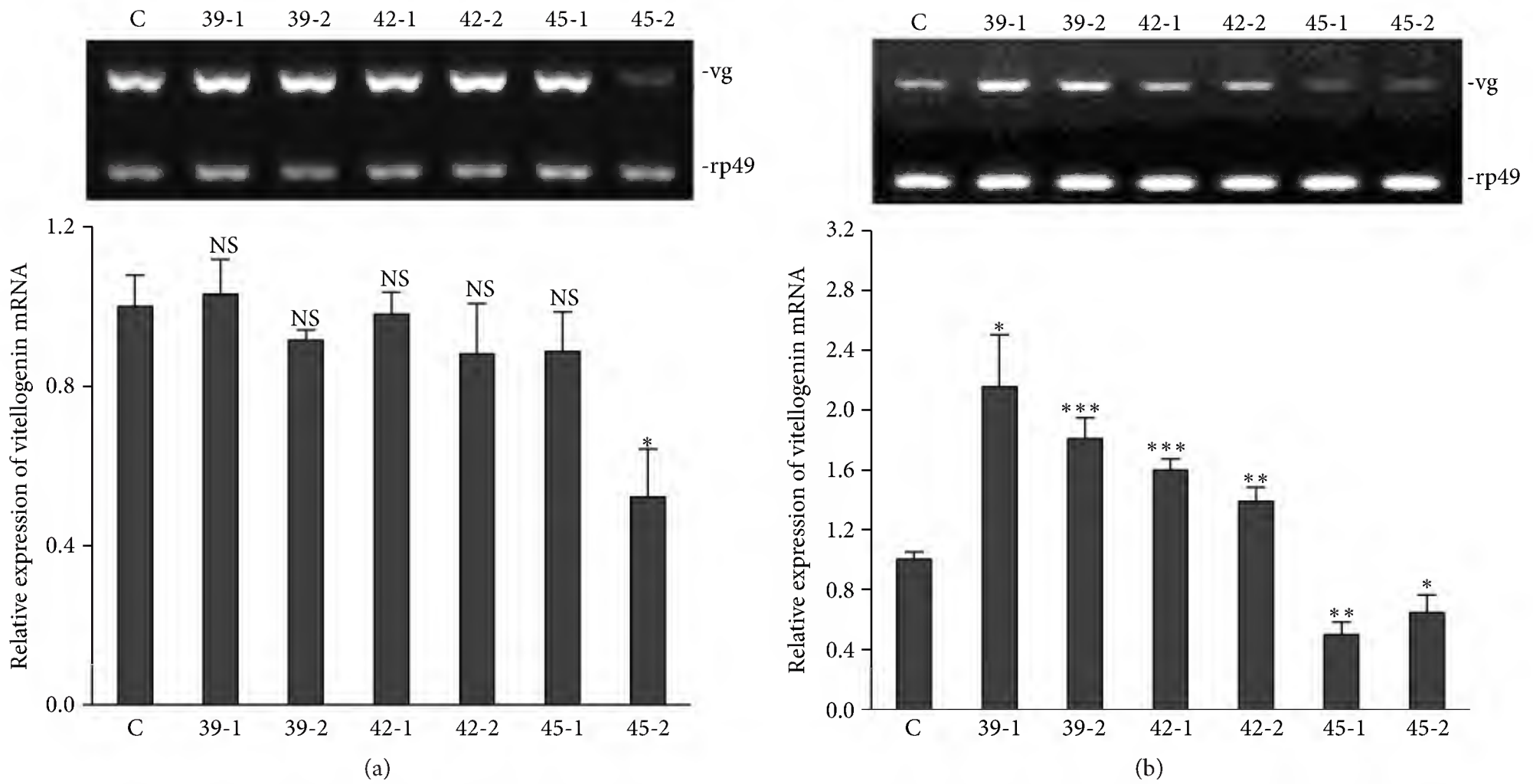


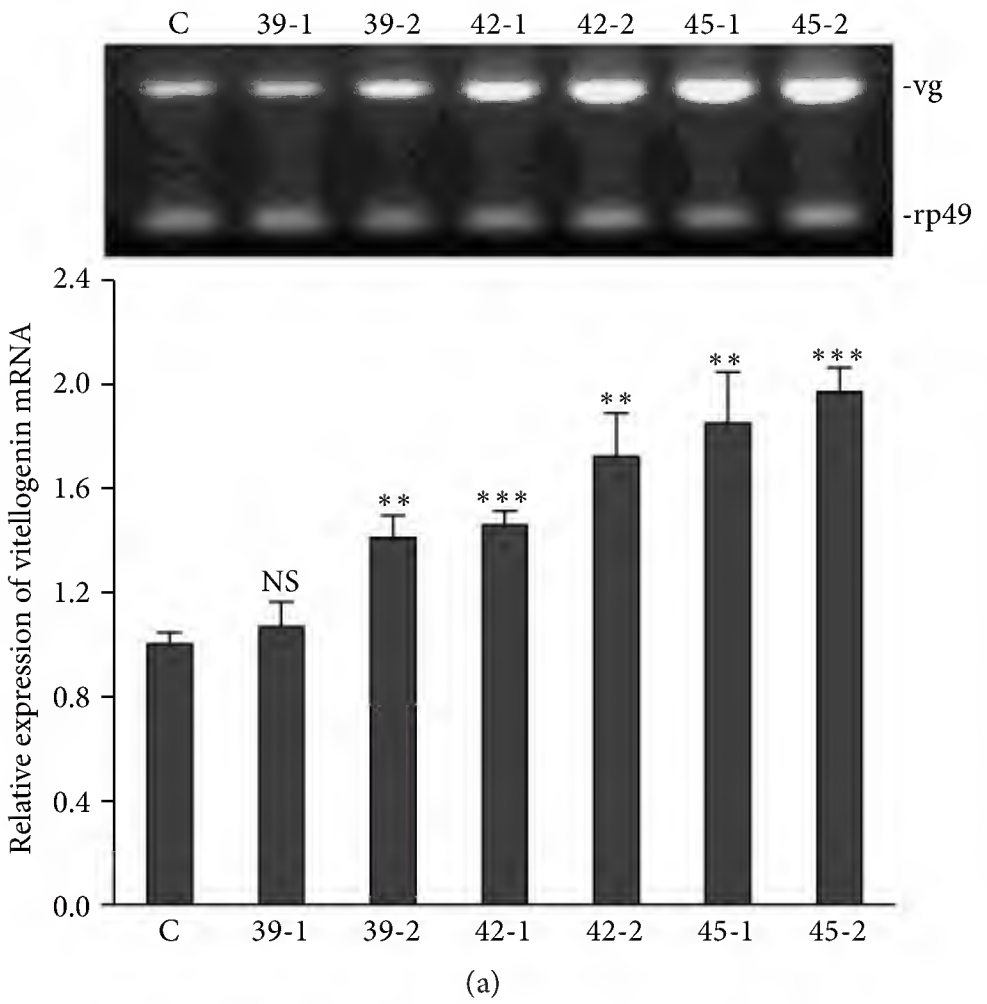
FIGURE 3: Vitellogenin mRNA expressions in spinning larvae upon heat shock. (a) Fat body. (b) Ovary. The upper panel showed expression of vitellogenin and rp49 as revealed by semiquantitative RT-PCR. The lower panel showed relative expression level of vitellogenin. The transcript level of the vitellogenin gene was calculated relative to *rp49* expression for each sample. Each histogram bar represents the mean relative expression of vitellogenin ($n = 5$–7) and the error bar represents standard error of the mean (SEM). The asterisks show the data which are significantly different from control sample (unpaired t-test; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). The abbreviations used are as mentioned for Figure 1.

no reproductive cost seems to be positively related with an increased expression of hsp90. Sarup et al. [40] suggested that the mild heat shock treatment might induce hormetic response as this treatment upregulates the synthesis of Hsps and extends the lifespan in Drosophila.

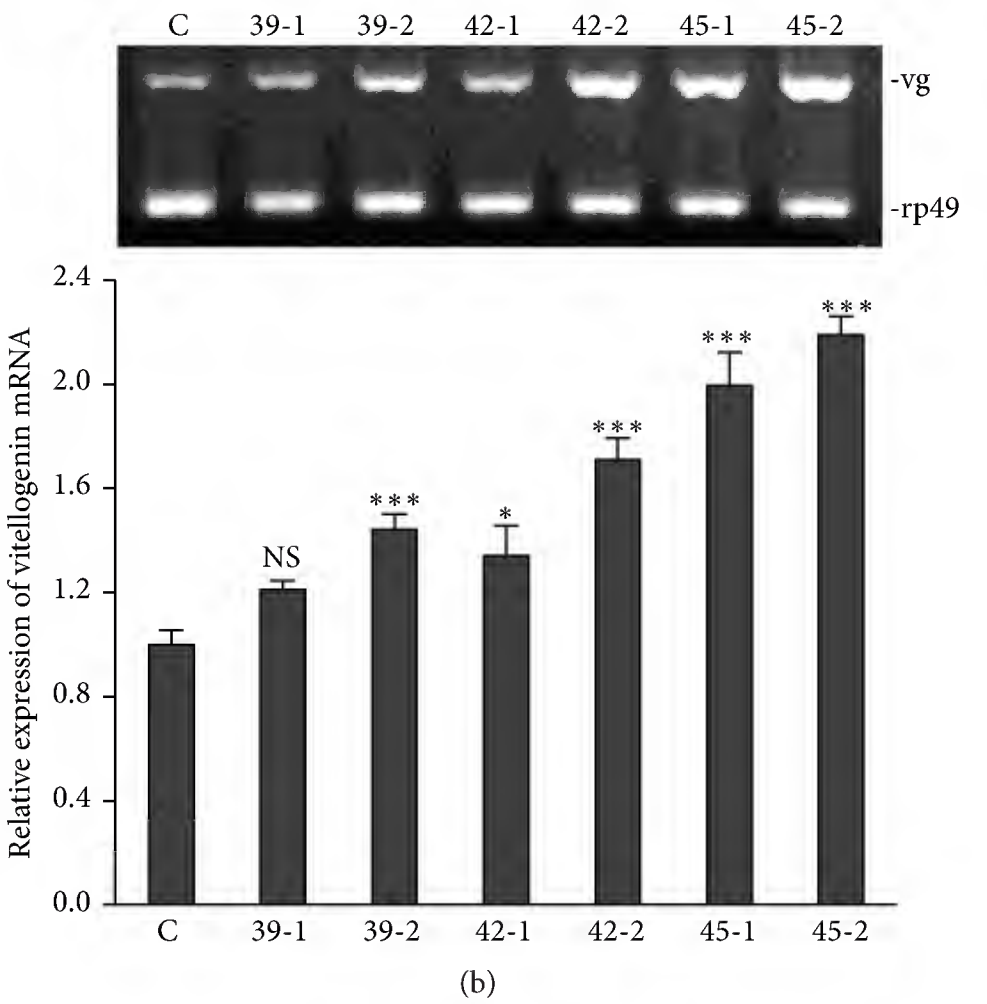
The heat shock treatment to *Bombyx* larvae and pupae at a temperature of 42° was not lethal for their survival, and majority of them after heat shock treatment entered into normal developmental program [25]. The heat shock at this temperature also induced the expression of hsp90 [25]. The present study showed that the heat shock exposure to Bombyx larvae and pupae at this temperature affects their ovarian development and reproductive performance as evident from decreased gonadosomatic index and fecundity. A "trade-off" thus exists between thermal resistance and reproduction in *B. mori*. In *D. melanogaster*, repeated mild heat stress caused increased thermotolerance but also brought a detrimental effect on fertility and fecundity [16]. In *D. virilis*, heat stress results in oocyte maturation delays, degradation of early vitellogenic egg chambers, and inhibition of yolk protein gene expression in follicle cells and accumulation of mature oocytes [41]. Heat shock also had deleterious effects on ovarian development in *Tribolium castaneum* [42], on fecundity in *Trialeurodes vaporariorum* and *Liriomyza huidobrensis* [27, 43], and on differentiation of the apyrene spermatozoa in *Bombyx* [44].

The vitellogenin mRNA showed a tissue- and developmental-specific expression upon heat shock treatment during larval spinning and pupal stages. In larval ovary, the heat shock at 39°C and 42°C caused an increase in vitellogenin mRNA expression, but with the increase of heat shock temperature to 45°C, a downregulation in its expression was observed. This pattern of upregulation of vitellogenin mRNA expression in larval ovary followed the same trend as was observed for hsp90 mRNA expression [25]. The induction of vitellogenin mRNA expression in larval ovary upon mild heat stress when the larvae could survive and molted to pupae indicates that vitellogenin might have a protective role in the survival of *B. mori* and thus suggests its role in hormesis like hsp90. The increased expression of vitellogenin might also help the differentiating tissues like ovary at spinning stage to cope up with the challenged situation, raised because of the heat shock treatment.

The data on the increased expression of vitellogenin mRNA upon heat shock treatment even at 45°C during pupal stage further support its protective action. It has to be noted that when heat shock was given at a temperature of 45°C, the pupae could survive for many days, although it could not eclose. An upregulation in the expression of vitellogenin with an increased magnitude of heat shock treatment is difficult to explain now but a detailed mechanistic study in future can explain the differential expression of vitellogenin upon heat shock. It is also difficult to hypothesize that the induction of vitellogenin upon heat shock treatment involves insect hormones, although it is quite known that the vitellogenesis in insects including *B. mori* is under the direct regulation of



(a)



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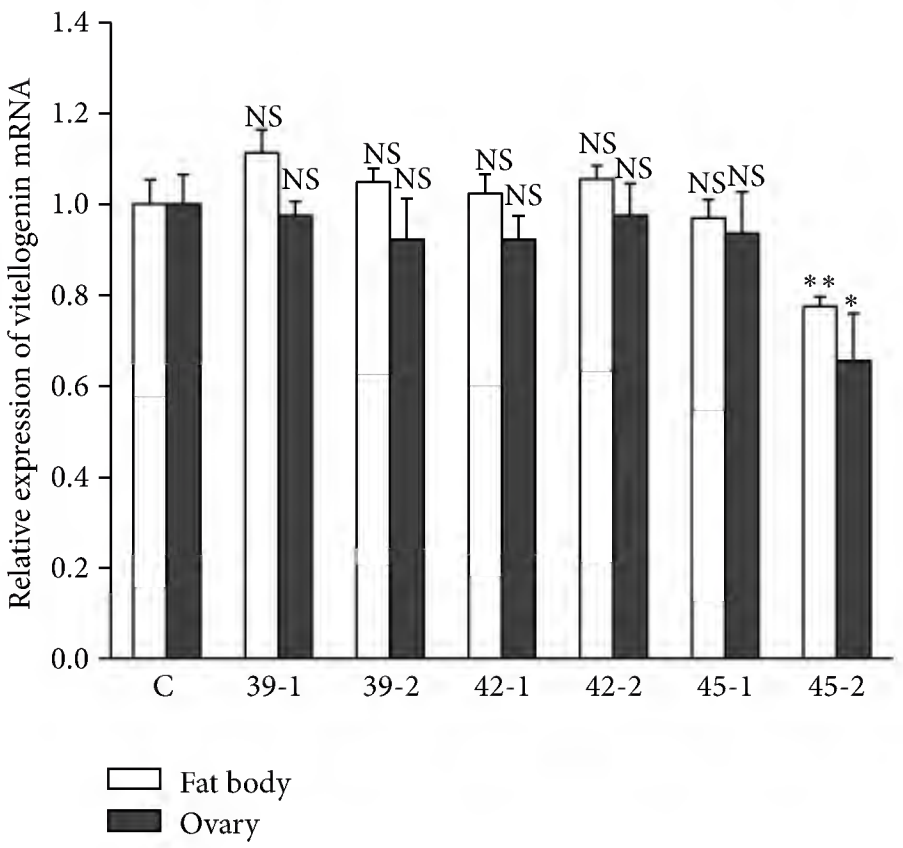

(c)

FIGURE 4: Vitellogenin mRNA expressions in pupae. (a) Fat body, day 2 pupae. (b) Ovary, day 2 pupae. (c) Fat body and ovary, day 4 pupae. The upper panel showed expression of vitellogenin and rp49 as revealed by semiquantitative RT-PCR. The lower panel showed relative expression level of vitellogenin as normalized with rp49 transcript level. Each histogram bar represents the mean relative expression of vitellogenin ($n = 5$–7) and the error bar represents standard error of the mean (SEM). The asterisks show the data which are significantly different from control sample (unpaired t-test; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). The abbreviations used are as mentioned for Figure 1.

insect hormones like juvenile hormone and/or ecdysteroids. Thus, the possibility remains that heat shock might have disrupted the normal hormonal milieu in *Bombyx* and thereby changed the vitellogenin expression in the fat body and ovary. Teixeira and polavarapu [45] showed that heat stress inhibits the completion of pupal diapause in *Rhagoletis mendax* and suggested that the failure to complete the development was due to the damage of the endocrine system by heat stress as the application of exogenous 20-hydroxyecdysone on these pupae caused them to develop to the adult stage.

The data further suggests that vitellogenin expression is temperature-dependent as in pupae, after the first significant increase as observed within 1 h of heat shock treatment, its expression returned to normal expression level at 48 h of heat shock treatment, except at 45°C, where a downregulation in its expression was observed. Thus, the

temperature-dependent variations in the expression of vitellogenin indicate its role in thermal protection of silkworm larvae and pupae besides its classical role as a precursor of egg yolk proteins. Although information regarding the other functional aspects of vitellogenin is not available much but limited literature showed its hemagglutinating and antibacterial activities [34]. The antibacterial activity of vitellogenin has been demonstrated in *Bombyx*, and it is shown that infected silkworm larvae, treated with vitellogenin, survived the full life cycle in contrast to untreated animals [35]. Vitellogenin also has the protective role against oxidative stress. It is synthesized at high levels in honey bee queens and is abundant in long-lived worker, protecting the sterile honey bee workers from oxidative stress [46]. Increased vitellogenin expression in *C. elegans* potently increases their resistance against heat [36]. Ihle et al. [37] showed that an experimental reduction of vitellogenin expression in honey bees resulted in decreased lifespan and increased susceptibility to oxidative damage.

In conclusion, the study hypothesizes that vitellogenin might have a protective role on the survival of silkworm. Further, a brief exposure of *Bombyx* larvae and pupae to a temperature of 42°C or higher (which is very common in tropical countries) greatly affects the ovarian development and reproductive performance of this commercially important insect. Further studies can be undertaken to know the mechanism of regulation of vitellogenin expression under heat stress in *B. mori* and other organisms and its protective role by interfering with its synthesis.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Terpenes: Natural Products for Controlling Insects of Importance to Human Health—A Structure-Activity Relationship Study

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Many insects affect food production and human health, and in an attempt to control these insects the use of synthetic insecticides has become widespread. However, this has resulted in the development of resistance in these organisms, human diseases, contamination of food, and pollution of the environment. Plants natural products and essential oil components such as terpenes and phenylpropenes have been shown to have a significant potential for insect control. However, the molecular properties related to their insecticidal activity are not well understood. The purpose of this review is to provide an overview of the toxicity of terpene compounds against three insects of importance to human health: lice, cockroaches, and Triatominae bugs and to evaluate which molecular descriptors are important in the bioactivity of terpenes. For the insects studied, quantitative structure-activity relationship (QSAR) studies were performed in order to predict the insecticidal activity of terpene compounds. The obtained QSAR models indicated that the activity of these compounds depends on their ability to reach the targets and to interact with them. The QSAR analysis can be used to predict the bioactivities of other structurally related molecules. Our findings may provide an important contribution in the search for new compounds with insecticidal activity.

1. Introduction

As many insects affect food production and human health, synthetic insecticides have often been used to control insects. However, an inappropriate use of these insecticides is linked to the development of resistance in pests, human diseases, and contamination of both the food and the environment [1–4]. Consequently, the biological action of natural products with insecticidal activity is a very important alternative, which allows an environment-friendly management of pest insects without affecting people's health.

Plants produce a wide diversity of compounds involved in their chemical defense. Among these natural products, terpene compounds have been shown to have a significant potential for insect control [1–5]. However, little is known about the molecular properties related to their insecticidal activity.

The quantitative structure-activity relationship (QSAR) is a mathematical expression by which the chemical structure is quantitatively correlated with well-known processes such as biological activity or chemical reactivity. In general, bioactivity studies using pure compounds tend to use molecules of different structures. Thus, for example, alcohols, ketones, and phenols with acyclic, monocyclic, or bicyclic structures are usually evaluated together. Although an initial study of bioactivities using a large number of molecules may provide some guidance on the chemical families of highest activity, not all the existing molecules can always be used, owing to practical and economic reasons. The use of molecules with high chemical diversity also causes problems for a QSAR analysis, as it is difficult to achieve a good correlation between the different physicochemical characteristics and bioactivities tested. This situation leads

us to select only certain molecules based on bibliographic information, causing an inherent limitation. Consequently, making an assessment using molecules of the same chemical family with small structural changes gives more appropriate information on the strength of these molecules as bioactive compounds than using a set of molecules that represent a large chemical diversity. Therefore, in our QSAR studies, a homologous series of compounds or derivatives of substances selected from bibliographic data were used, which reduced the variability of topological and electronic features that results from using a variety of structures and hinders the regression analysis when obtaining a predictive model. A QSAR study shows the connection between a set of predictor variables (chemicals) and the response variable (biological activity) of the chemicals. QSAR models create a relationship between chemical structures and biological activity in a data set of chemicals. However, as limited bibliographic information was obtained from the QSAR studies with pest insects, in the present study, we used the results of some investigations related to this issue. Furthermore, we performed QSAR models on the physicochemical descriptors of the terpene compounds and their insecticidal activities against three insects of importance to human health, namely, lice, cockroaches, and Triatominae bugs (vector of Chagas disease).

2. Molecular Modelling, Calculation of Molecular Parameters, and Statistical Analysis

The bioactivities of natural compounds were selected from bibliographic data, and the physicochemical, electronic, and topological descriptors of these molecules were used for the QSAR analysis.

The descriptors used for each molecule were octanol/water partition coefficient (log P), molar refractivity, molar volume, parachor, index of refraction, surface tension, density, polarizability, polar surface area (pH 7.4), molecular surface area, and solvent-accessible surface area of all polar atoms, which were calculated using ChemAxon Soft (Cambridge Soft Corp.) [6]. Vapor pressure, boiling point, hydrogen bond donors, hydrogen bond receptors, vapor density, enthalpy of vaporization, and dipole were obtained by using ChemSpider 2014 [7].

A multiple linear regression analysis (MLR) was performed in order to examine the quantitative relationships between linear combinations of the dependent variable (log (1/bioactivity) or LN (inhibition%)) with the predictor variables (structure and molecular properties). In the MLR equations, N is the number of data points, R is the correlation coefficient between observed values of the dependent variable and the values calculated from the equation, and R^2 is the square of the correlation coefficient and represents the goodness of fit. The obtained QSAR model was validated using the root mean square prediction error (RMSPE), obtained by cross validation leave-one-out procedure. Results with P values < 0.05 were considered to be significant. All statistical analyses were performed using the InfoStat software Professional 2010 p [8].

3. Lice

Lice infestation or pediculosis is a global problem. Usually, the lice that infest humans are sucking lice that live in close association with the host and lay their eggs on hair shafts or in the seams of clothing [9]. *Pediculus humanus humanus* (Linnaeus 1758) and *Pediculus humanus capitis* (De Geer 1767) are two obligate ectoparasites which affect the body and head of the host, respectively, with *Pediculus humanus* appearing to be the vector of *Rickettsia prowazeki* and *Borrelia recurrentis,* two microorganisms of importance to human health [10–12]. The management of head lice infestation has a major disadvantage compared to the control of other insect pests, as the human hosts want the head lice to be completely eliminated [13]. However, the search to achieve total elimination and the use of inappropriate chemical controls have allowed head lice to develop resistance [14] to a significant number of pesticides such as lindane, DDT, carbaryl, malathion, and pyrethroids [11, 15, 16]. Consequently, the use of naturally occurring insecticidal compounds could be an attractive alternative to control head lice, including those which are resistant to pesticides [14]. Many scientific studies have proposed the use of various natural compounds, with essential oils having been shown to have different activities such as repellent, ovicidal, and adulticide properties in a variety of insect species, including head lice [3, 11, 13, 15–27]. Essential oils and terpenes have also been used in combination with conventional insecticides, and the results of these experiments have demonstrated an increase in the pediculicide properties of synthetic compounds [15, 28]. Nevertheless, one problem in controlling head lice with essential oils is the variability present in their composition, which can be observed within a species because of environmental conditions (soil, moisture, nutrients, etc.), and it prevents a standard formulation being effective for the control of head lice. Thus, pure compounds of essential oils should be used in the control of body and head lice.

3.1. Terpenes against Adult Head Lice. Essential oils have shown different results as pediculicidal agents [3, 11, 20, 22, 24–26, 28–33]. Among the components of essential oils, 1,8-cineole, anisole, limonene, β-pinene, linalool, menthone, α-pinene, pulegone, and myrcene have demonstrated fumigant activity ($KT_{50} < 53$ min) whereas eugenol, borneol, menthol, isomenthol, anethole, camphor, carvone, menthyl acetate, linalyl acetate, thymol, p-cymene, γ-terpinene, α-terpineol, and 4-terpineol had no effect ($KT_{50} > 60$ min) [3, 11, 13, 19, 20]. Moreover, Gonzalez-Audino et al. [34] and Gallardo et al. [35] reported that citronellol and geraniol had the highest toxicity on adults for immersion and topical application methods, respectively.

It has been suggested that the mechanism of action of essential oil components could be due to competitive inhibition of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7). Some researchers indicated that ketone compounds

Table 1: Calculated molecular descriptors[a] for terpene and phenylpropene compounds used in QSAR analysis of the fumigant activity against head lice.

Compounds[b]	MV	PSA	BP	HBA
1,8-Cineole	167.1	9.23	177	2
Anisole	113.4	9.23	155	2
Anethole	154.4	9.23	237.5	2
Eugenol	156.2	29.46	252.5	4
Thymol	154.2	20.23	231.5	2
Linalool	179.6	19.62	199	2
Benzyl alcohol	103.2	20.23	205	2
(+)-Borneol	155.3	20.23	212.5	2
Citronellol	184.9	20.23	225	2
Menthol	175.5	20.23	216	2
Isomenthol	175.5	18.96	218.5	2
Camphor	154.8	17.59	204.5	1
Carvone	159.7	18.03	230.5	2
Geraniol	177.9	19.7	230	2
Menthone	175	17.07	209.5	2
Pulegone	164.8	17.07	221	2
Limonene	163.2	0	176	0
α-Pinene	154.9	0	156	0
β-Pinene	153	0	165	0
Myrcene	177	18.85	167	0
γ-Terpinene	161.1	18.01	182	0

[a]The descriptors were calculated by ChemAxon and ChemSpider software.
[b]The data on lice were obtained by Toloza [13].

augment the inhibitory effect on AChE because of the presence of the double bond of the carbonyl group [36, 37]. Picollo et al. [38] showed that two ketones, menthone and carvone, significantly inhibited AChE activity in head lice, but another study reported that carvone did not have a good fumigant activity against lice [11]. Other terpenes and phenylpropenes that have demonstrated inhibitory effects on AChE of head lice are 1,8-cineole and eugenol, while isomenthol and menthol were reported not to have any inhibitory activity [38].

The use of essential oils permits interaction with different targets simultaneously as a result of chemical diversity, although chemical diversity of an essential oil with a high bioactivity is difficult to reproduce. An artificial mixture of terpenes or phenylpropene has demonstrated a high activity and could have synergistic effects [35, 39], with linalyl acetate mixed with other terpenes showing a better synergistic effect on the inhibition of AChE activity than many isolated compounds.

We performed a multiple linear regression analysis to determine the mathematical function that could best predict the fumigant activity of some oxygenated terpenes, namely, 1,8-cineole, anisole, anethole, eugenol, thymol, linalool, benzyl alcohol, borneol, citronellol, menthol, isomenthol, camphor, carvone, geraniol, menthone, and pulegone, with the calculated molecular descriptors used in the analysis being shown in Table 1. The obtained model was statistically significant ($P = 0.0001$), and it predicted KT_{50} (min^{-3}) based on four molecular properties, namely, boiling point (BP), polar surface area (PSA), molar volume (MV), and hydrogen bond acceptor (HBA):

$$\log\left(\frac{1}{KT_{50}}\right) = 0.23\,(HBA) + 0.0023\,(MV) - 0.01\,(BP) - 0.01\,(PSA) + 2.77, \quad (1)$$

$$N = 16;\ R^2 = 0.96;\ \text{RMSPE} = 10.5\%;\ P < 0.0001.$$

A second QSAR analysis was performed in order to include the terpene hydrocarbons limonene, α-pinene, β-pinene, myrcene, and γ-terpinene in the model. The obtained model was statistically significant ($P = 0.0001$), and it predicted the toxicity (KT_{50}) of the terpenes (in (2)) based on boiling point (BP), polar surface area (PSA), and hydrogen bond acceptor (HBA). The incorporation of hydrocarbon compounds induced a decrease of model fit, suggesting that the use of homologous series reduces the variability features that hinder regression analysis. The resulting equation was of the following form:

$$\log\left(\frac{1}{KT_{50}}\right) = 0.01\,(BP) - 0.01\,(PSA) + 0.2\,(HBA) + 2.82, \quad (2)$$

$$N = 21;\ R^2 = 0.81;\ \text{RMSPE} = 7.3\%;\ P < 0.0001.$$

The analysis of compounds of different chemical natures can cause a loss of fit of the QSAR model (in (2)) and therefore can lead to conflicting conclusions. For example, according to model 1, oxygenated terpenes of lower boiling points should be more active, while, according to model 2, these should be less active. Also, the plot of calculated versus experimental $\log(1/KT_{50})$ is shown in Figures 1 and 2, with HBA being an important descriptor in both formulas. Thus, an increase in the value of HBA represents a greater insecticidal activity. However, it is known that hydrocarbons have no HBA, and hence this descriptor in (2) would represent a dipole-dipole interaction between the hydrocarbons and target instead of HBA.

3.2. Ovicidal Effects. One way to control head lice infestation is by reducing the development of their eggs. Bibliographic information shows some components of essential oils having a high ovicidal activity, such as methyl salicylate, eugenol, linalool, terpinen-4-ol, carveol, geraniol, nerolidol, thymol, α-terpineol, 1,8-cineole, linalool, carvone, anethole, anisole, α-pinene, β-pinene, limonene, salicylaldehyde, benzaldehyde, cinnamaldehyde, and benzyl cinnamate [13, 29–31, 33]. However, other studies did not reveal any ovicidal activity in the following compounds: menthol, isomenthol, nonyl alcohol, benzyl alcohol, citronellol, geraniol, camphor, menthone, eugenol, thymol, terpinene and acetate esters of menthol and linalool, acetyleugenol, caryophyllene, humulene, isoeugenol, methyl eugenol, 1-R-terpineol, (E)-pinocarveol,

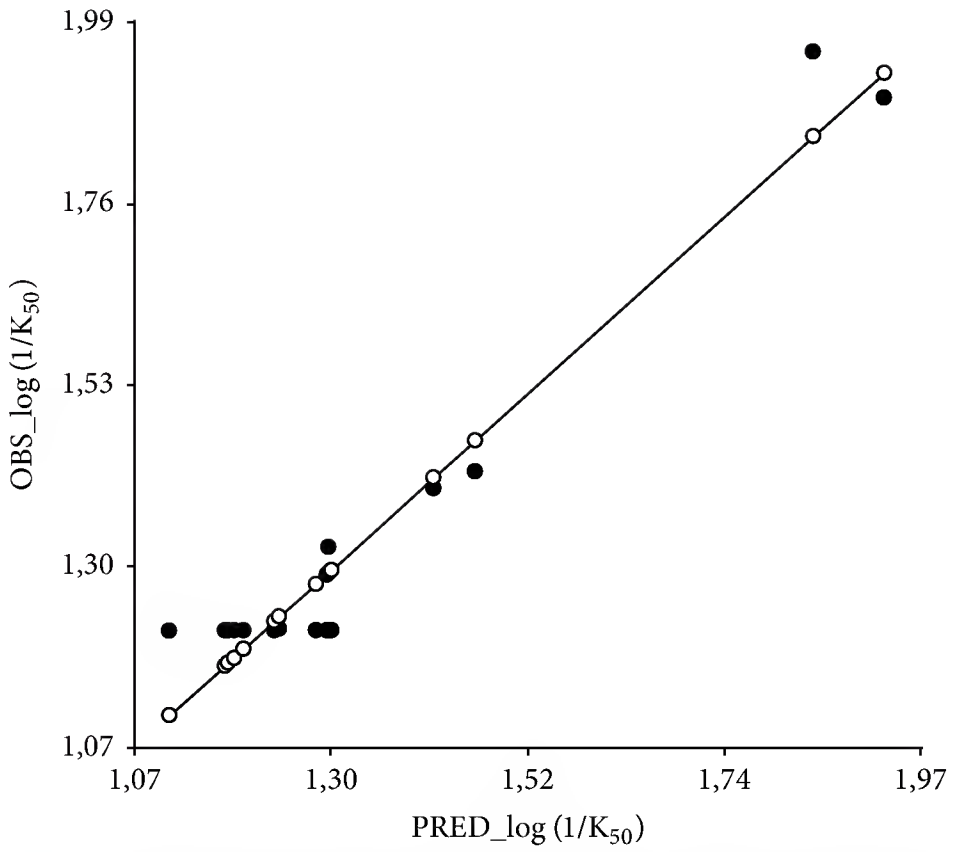

Figure 1: Multiple linear regression analysis (MLR) calculated to examine quantitative relationships between linear combinations of the dependent variable $\log(1/KT_{50})$ and the predictor variables (structure and molecular properties). Plot of calculated versus experimental $\log(1/KT_{50})$ of fumigant activity of oxygenated terpenes against lice.

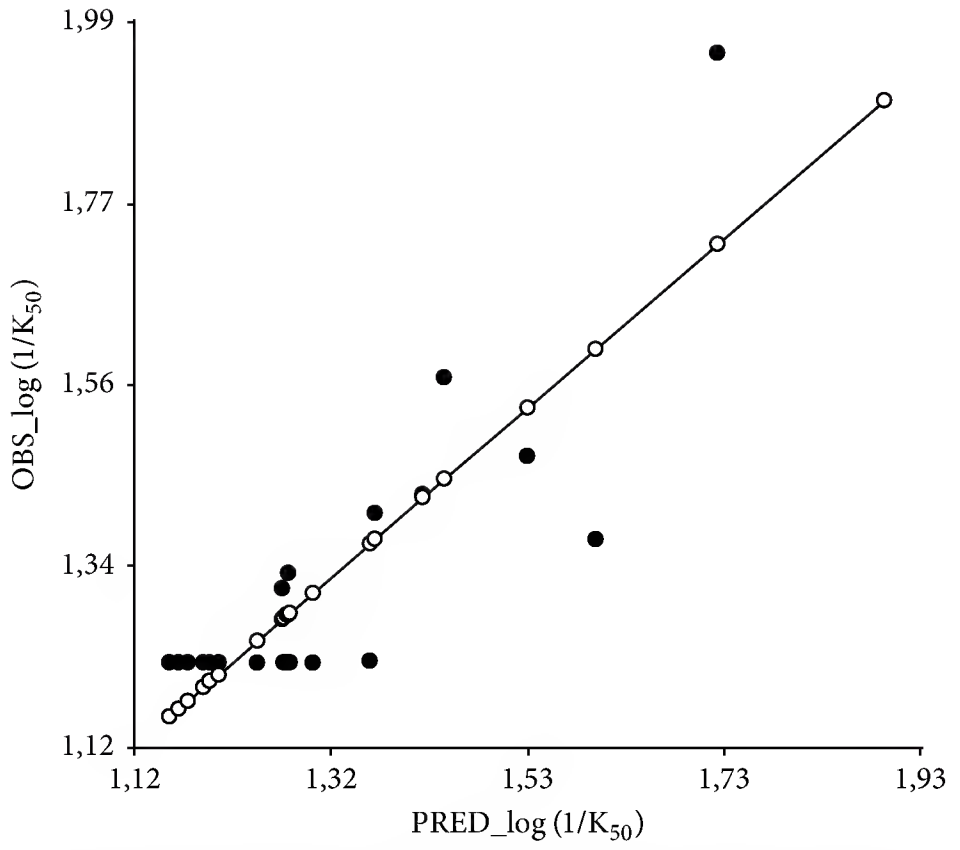

Figure 2: Multiple linear regression analysis (MLR) calculated to examine quantitative relationships between linear combinations of the dependent variable $\log(1/KT_{50})$ and the predictor variables (structure and molecular properties). Plot of calculated versus experimental $\log(1/KT_{50})$ of fumigant activity of oxygenated and hydrocarbon terpenes against lice.

salicylaldehyde, benzaldehyde, cinnamaldehyde, benzyl cinnamate, benzyl alcohol, limonene, α-pinene, β-pinene, γ-terpinene, α-terpinene, 1,8-cineole, and cinnamyl acetate [29–31]. Moreover, from bibliography, it can be seen that there are conflicting results regarding the efficiency of essential oil components for lice control, possibly related to the different techniques used for evaluating the bioassays. It has also been suggested that pediculicidal activity developed by the components of essential oils also depends on the *Pediculus* species [13, 29–31].

In this section, the order of toxicity of 20 natural components of essential oils to ovicidal activity against *Pediculus humanus* capitis is examined using data previously reported by Toloza [13]. These results show that the ether terpenes 1,8-cineole, anisole, and anethole and also the hydrocarbon terpenes limonene, α-pinene, β-pinene, and γ-terpinene were the most active compounds evaluated. A QSAR analysis was performed in order to determine the mathematical function that best fitted or predicted the activity of alcohol and the ketone terpenes. However, the most active terpenes (ether and hydrocarbon) were not included in this QSAR analysis, because they increased the chemical diversity and thereby influenced the fit obtained in the model. On the other hand, the ether and hydrocarbon terpenes were not sufficient to perform a QSAR analysis.

The calculated molecular descriptors used in the analysis are shown in Table 2. The model obtained using alcohols and ketones is given by the following expression:

$$\begin{aligned} &\mathrm{LN\,(Inhibition\%)} = -0.011\,(\mathrm{BP}) + 26.10, \\ &N = 9;\ R^2 = 0.84;\ \mathrm{RMSPE} = 15.4\%;\ P = 0.0013. \end{aligned} \tag{3}$$

Table 2: Calculated molecular descriptors[a] for alcohol and ketone compounds used in QSAR analysis of the ovicidal activity against *Pediculus humanus capitis*.

Compounds[b]	BP
Linalool	198.5
Benzyl alcohol	204.7
Menthol	215.3
Isomenthol	215.4
Geraniol	229.49
Menthone	205
Camphor	207.4
Nonyl alcohol	211.6
Citronellol	224.5

[a]The descriptors were calculated by ChemAxon and ChemSpider software.
[b]The data on lice were obtained by Toloza [13].

The negative coefficients of BP indicate that an increase in this descriptor led to a decrease in ovicidal toxicity, with this QSAR model showing a good correlation between the estimated and experimentally measured toxicity parameters for the tested monoterpenes and phenylpropenes. This result is in agreement with the high activity of ethers and hydrocarbons terpenes reported by Toloza [13], because these are the most volatile compounds evaluated in the cited work. A plot of the calculated versus experimental LN (inhibition%) is shown in Figure 3. Developing these types of relationships may facilitate the design of more effective ovicidal components and provide an insight into the structural properties that are responsible for their toxicities.

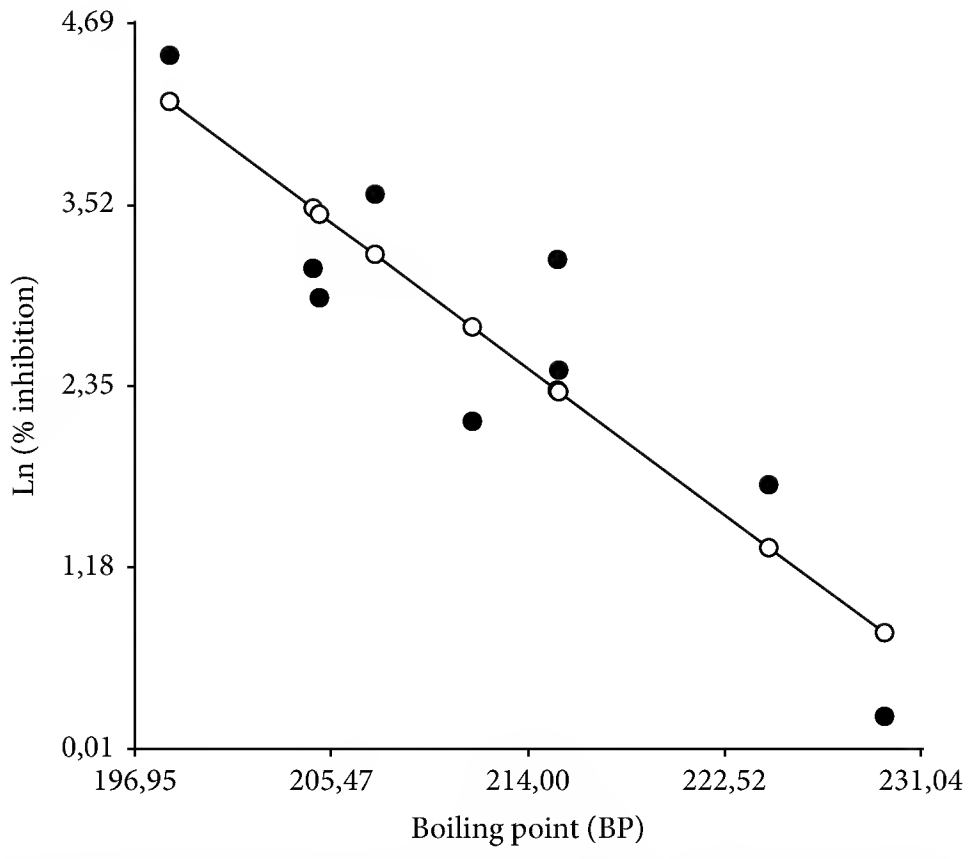

FIGURE 3: Multiple linear regression analysis (MLR) calculated to examine quantitative relationships between linear combinations of the dependent variable LN (Inhibition%) and the predictor variables (structure and molecular properties). Plot of calculated versus experimental LN (Inhibition%) of ovicidal activity of alcohol and ketone terpenes against lice.

3.3. Repellent. Repellency can help describe the behavioral effects that alter the perception of the peripheral nervous system (PNS), which leads to the insect neither biting nor sucking and causes them to move away from their food source [40]. Thus, repellency is a strategy to prevent reinfection of host by an ectoparasite but does not result in the death of the insect. Essential oils have developed a very important role as natural repellent agents [13, 15, 41], with the results of the previously reported studies showing that benzyl alcohol was the most effective compound with a repellent index of 57.74%, followed by (−)-menthol, menthone, and (+)-menthol, with RI values of 53.56, 39.23, and 36.45%, respectively.

Toloza et al. [11] were the first to report on the potential of acyclic lactones as head lice repellents, with the repellent activity of these lactones ranging from 60 to 76% (equivalent to piperonal repellent) and δ-dodecalactone being the most active (76.68%). In addition, the lactone compounds were more active than the terpene ones. In fact, the δ- and γ-lactones are common components in the aroma of coconut oil.

4. Cockroaches

The term "cockroaches" covers a large number of species which have a worldwide distribution, with these insects being considered to be excellent transporters of pathogenic and allergic substances of faecal origin [42, 44]. However, as occurs in the control of other pests, the frequent use of synthetic insecticides has also generated resistance in cockroaches. Moreover, the enormous potential toxicity of synthetic insecticides has led to their use being restricted in establishments where food is prepared, such as in the hospitals [42]. As mentioned above, the new phytoinsecticides can be applied in both open and closed places, in the same way as synthetic insecticides, with essential oils and their components being considered minimum risk pesticides [45].

Several studies have reported insecticidal activities of terpenes against cockroaches. An important aspect in the evaluation of phenylpropene or terpenes as insecticides against cockroaches is the sex of the insect, and the results of different investigations have shown the necessity of higher doses of the insecticide for females [46–49]. As cockroach females have a higher body lipid concentration than males, this situation may be generating a differential effect on the actions of essential oils. Also, males are more sensitive to the insecticidal action of terpenes. Thus, the higher concentration of lipids in the female body might be trapping the terpenes and thereby avoiding their insecticidal action [47]. However, there are other factors that differentially affect males and females in insecticidal bioassays. For example, thymol, trans-cinnamaldehyde, 1,8-cineole, and menthone terpenes have been demonstrated to have different sensitivities between males and females [46, 48].

The experimental results reported by Jang et al. [42] were now used to perform a quantitative structure-activity analysis, with the molecular descriptors used in the QSAR studies being listed in Tables 3, 4, and 5.

4.1. Contact Toxicity Bioassay. In the contact assay, the values of LC_{50} (24 h exposure) in female *B. germanica* revealed the adulticidal activity of pulegone (0.06 mg/cm^2), camphor (0.07 mg/cm^2), and verbenone (0.07 mg/cm^2), with similar results for camphor and pinene isomers having been reported by Jung et al. [50]. In contrast, linalool, terpineol, thymol, perillaldehyde, 1,8-cineole, and thujone showed a range of adulticidal activities between 0.09 and 0.18 mg/cm^2 [42]. The insecticidal activity of pulegone may also be explained by its interaction with the octopamine receptor. Pulegone, p-cymene, trans-anethole, vanillin, and isoeugenol are terpenes and phenylpropenes which exert strong octopaminergic receptor antagonist activity at concentrations of 2 nmol/mL [51]. The lower insecticidal activity of linalool and terpineol could be due to their lack of interaction with the octopamine receptor [42]. In the case of terpineol, the binding activity of octopamine receptors was blocked with an approximate IC_{50} value of 9 nmol/mL [51].

Yeom et al. [49] showed that eugenol, isoeugenol, methyl eugenol, terpinen-4-ol, carveol, cuminaldehyde, (S)-(+)-carvone, trans-anethole, thymol, and p-cymene had a strong activity at 1 mg/adult in a topical application bioassay.

Two QSAR models were utilized for evaluating the contact toxicity assay:

(i) "Alcohols and phenols" (thymol, α-terpineol, linalool, verbenol, menthol, carvacrol, (−) carveol, terpinen-4-ol, borneol, citronellol, geraniol, and cinnamyl alcohol)

(ii) "Aldehydes and ketones" (pulegone, camphor, verbenone, α-thujone, 1,8-cineole, perillaldehyde, cinnamaldehyde, menthone, carvone, and citronellal)

Table 3: Calculated molecular descriptors[a] for alcohol and phenol compounds used in QSAR analysis of the insecticidal activity against cockroach species.

Compounds[b]	Rings	pka	MV	Parachor	RI	ASA	ASAH	ASAP
Thymol	1	10.59	154	374.9	1.52	356.3	329.7	26.6
α-Terpineol	1	19.4	164.9	396	1.48	335.6	318.4	17.2
Linalool	0	18.46	179.6	414.2	1.46	378.4	358.1	20.2
Verbenol	2	18.59	151.8	362.5	1.51	290	266.4	23.6
Menthol	1	19.55	175.5	409.8	1.46	336.9	313.8	23.1
Carvacrol	1	10.42	154.2	374	1.52	364	328.9	35.1
(−)-Carveol	1	18.21	160.2	383.1	1.5	313.8	289	24.3
Terpinen-4-ol	1	20	165.2	396	1.48	317.5	301	16.5
Borneol	2	19.6	155.3	381.2	1.5	273.6	250.7	22.9
Citronellol	0	17.11	184.9	427.5	1.45	419.1	378.6	40.5
Geraniol	0	16.33	177	413.5	1.47	411.6	367.5	44
Cinnamyl alcohol	1	15.62	127.9	326.9	1.56	328.8	284.1	44.7

[a]The descriptors were calculated by ChemAxon and ChemSpider software.
[b]The data on cockroach species were obtained by Jang et al. [42].

Table 4: Calculated molecular descriptors[a] for aldehyde and ketone compounds used in QSAR analysis of the insecticidal activity against cockroach.

Compounds[b]	MV	Parachor	RI	ST	ASA	ASA+	ASA−	Vp	BP
Pulegone	164.8	384.2	1.47	29.5	347	249.8	97.2	0.09	224
Camphor	154.8	367.1	1.49	31.5	277.6	212	65.6	0.23	207.4
Verbenone	151.4	352.7	1.49	29.4	293.7	210.5	83.3	0.08	227.5
α-Thujone	150.8	366.8	1.5	34.9	318	236.3	81.7	0.32	200.5
1,8-Cineole	167.1	399.1	1.46	32.4	307.7	242.4	65.3	1.65	174
Perillaldehyde	149.8	377.1	1.54	40.1	317.1	237.2	79.9	0.04	238
Cinnamaldehyde	127.7	319.1	1.58	38.9	299.2	210.3	88.9	0.03	246.8
Menthone	175	400	1.44	27.2	346.6	262.6	84.1	0.26	205
Carvone	159.7	373.3	1.48	29.8	302.7	233.3	69.3	0.07	230.5
Citronellal	184.7	419.6	1.44	26.6	401.5	282.3	119.1	0.21	208.4

[a]The descriptors were calculated by ChemAxon and ChemSpider software.
[b]The data on cockroach species were obtained by Jang et al. [42].

The model obtained for group (i) is given by the following expression:

$$\log\left(\frac{1}{LC_{50}}\right) = -0.09\,(\text{ASA P}) - 0.81\,(\text{rings}) - 0.08\,(\text{Parachor}) - 46.67\,(\text{RI}) - 0.17\,(\text{Pka}) + 108.7, \quad (4)$$

$$N = 12;\ R^2 = 0.98;\ \text{RMSPE} = 17.6\%;\ P = 0.002;$$

and the model obtained for group (ii) is given by the following expression:

$$\log\left(\frac{1}{LC_{50}}\right) = 0.25\,(\text{ST}) + 0.02\,(\text{ASA}) - 52.29\,(\text{RI}) - 2.33\,(\text{VP}) - 0.05\,(\text{ASA+}) - 0.05\,(\text{MV}) + 85.74, \quad (5)$$

$$N = 10;\ R^2 = 0.97;\ \text{RMSPE} = 8.2\%;\ P = 0.0028.$$

As the analysis of monoterpene hydrocarbons did not adjust properly to the quantitative structure-activity analysis, this QSAR model was not reported. The plots of calculated versus experimental log ($1/LC_{50}$) are shown in Figures 4 and 5.

Equations (4) and (5) revealed that the index of the refraction or refractive index (RI) of the alcohol-phenol and aldehyde and ketone compounds negatively influences the lethal concentration 50 (LC_{50}), with the polarizability of the molecule being related to RI. In contrast, in (5), a rise in the vapor pressure (VP) of terpenes elevates the lethal concentration 50 (LC_{50}).

4.2. Fumigant Toxicity Bioassay. The results published in several scientific studies have shown that the presence of a carbonyl group in the terpene is important, which can act as an acceptor of hydrogen bonding. This may permit the terpene to interact with water molecules on the surface of the insect respiratory system (tracheoles) and consequently provides a better lead towards the target site [42, 52]. When evaluating the terpenes as fumigants, a mechanism of action

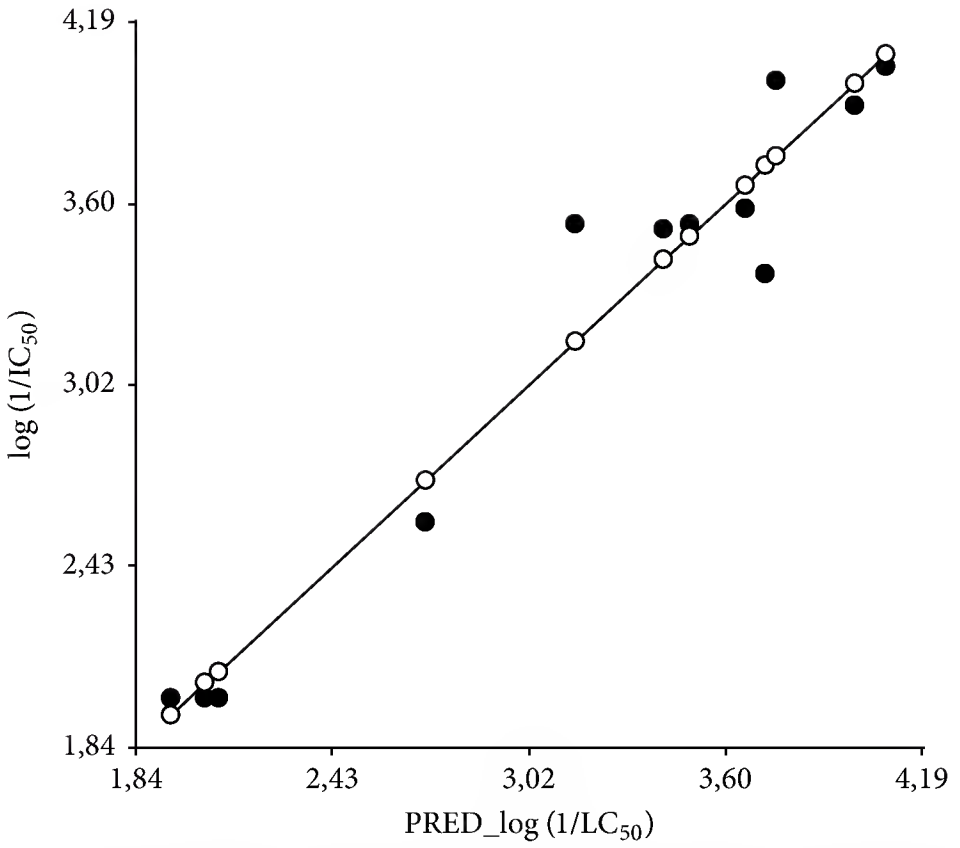

FIGURE 4: Multiple linear regression analysis (MLR) calculated to examine quantitative relationships between linear combinations of the dependent variable log (1/LC_{50}) and the predictor variables (structure and molecular properties). Plot of calculated versus experimental log (1/LC_{50}) of repellent activity of alcohol and phenol compounds against cockroaches.

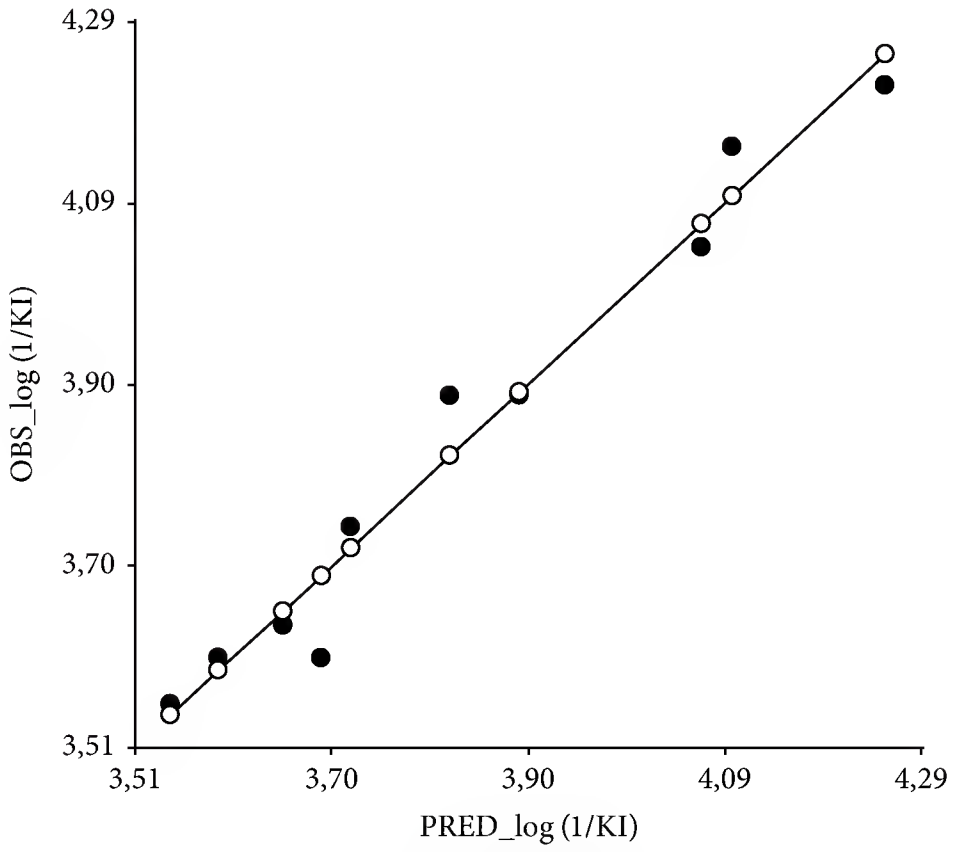

FIGURE 5: Multiple linear regression analysis (MLR) calculated to examine quantitative relationships between linear combinations of the dependent variable log (1/LC_{50}) and the predictor variables (structure and molecular properties). Plot of calculated versus experimental log (1/LC_{50}) of repellent activity of aldehyde and ketone compounds against cockroaches.

similar to the carbonyl group can be proposed for ether such as 1,8-cineole [46].

The interaction of carbonyl groups of terpenes with the GABA receptor may be related to the fast knockdown of insects as a result of their interaction with the GABA receptors present in the insect neuromuscular junctions [53].

TABLE 5: Calculated molecular descriptors[a] for hydrocarbon compounds used in QSAR analysis of the fumigant efficacy against cockroach.

Compounds[b]	Rings	MV	log P
α-Phellandrene	1	163.135	3.21
2-Carene	2	154.905	2.8
γ-Terpinene	1	161.128	3.16
Camphene	2	153.074	2.86
β-Pinene	2	153.074	2.86
3-Carene	2	154.905	2.8
Limonene	1	163.264	3.22
α-Terpinene	1	161.128	3.16
α-Pinene	2	154.905	2.8
Myrcene	0	177.007	3.54

[a]The descriptors were calculated by ChemAxon and ChemSpider software.
[b]The data on cockroach species were obtained by Jang et al. [42].

In contrast, thujone does not have a similar activity to other ketones, due to the presence of a cyclopropane in its structure, according to Tong and Coats [54].

Zhu et al. [55] reported that (Z)-ascaridole, isoascaridole, and p-cymene possessed fumigant toxicity against male German cockroaches. Other monoterpenes with observed fumigant activity include terpinolene, α-terpinene, terpinen-4-ol, (S)-(+)-carvone, 1,8-cineole, trans-dihydrocarvone, cuminaldehyde, trans-anethole, p-cymene, and γ-terpinene [49, 56]. In addition, fumigant activity against *B. germanica* has been reported with carvacrol [57, 58], (E)-anethole [59], and thymol [42], with derivatives of the biosynthetic pathway of shikimic acid, such as isosafrole and safrole, having shown adulticidal activity against the female *Periplaneta americana* (L.) [60].

The QSAR studies for fumigant toxicity assay were represented by three models:

(i) "Alcohols and phenols" (thymol, α-terpineol, linalool, verbenol, menthol, carvacrol, (−)-carveol, terpinen-4-ol, borneol, citronellol, geraniol, and cinnamyl alcohol)

(ii) "Aldehydes and ketones" (pulegone, camphor, verbenone, α-thujone, 1,8-cineole, perillaldehyde, cinnamaldehyde, menthone, carvone, and citronellal)

(iii) "Hydrocarbons" (α-phellandrene, 2-carene, γ-terpinene, camphene, β-pinene, 3-carene, limonene, α-terpinene, α-pinene, and myrcene)

The algorithm for models (i), (ii), and (iii) is expressed in (6), (7), and (8), respectively:

$$\log\left(\frac{1}{LD_{50}}\right) = 0.10\,(\text{ASA H}) - 0.08\,(\text{ASA}) - 0.03\,(\text{MV}) + 5.15, \quad (6)$$

$$N = 12;\ R^2 = 0.95;\ \text{RMSPE} = 8.6\%;\ P < 0.0001,$$

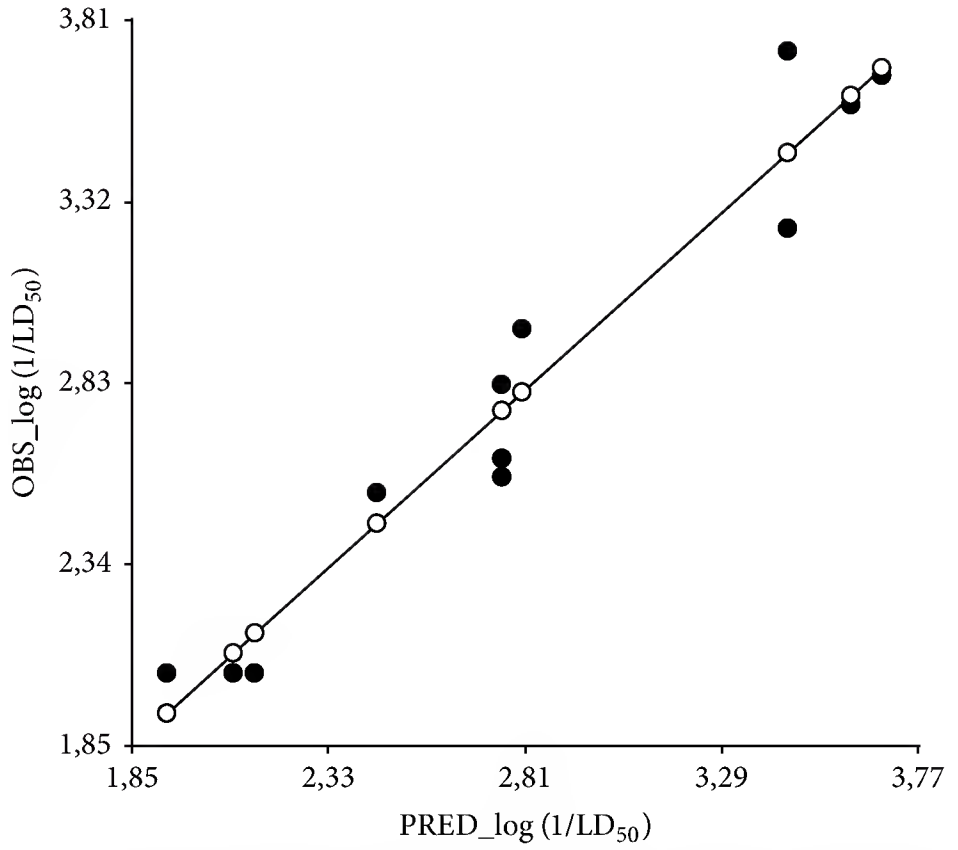

Figure 6: Multiple linear regression analysis (MLR) calculated to examine quantitative relationships between linear combinations of the dependent variable $\log(1/LD_{50})$ and the predictor variables (structure and molecular properties). Plot of calculated versus experimental $\log(1/LD_{50})$ of insecticidal activity of alcohol and phenol compounds against cockroaches.

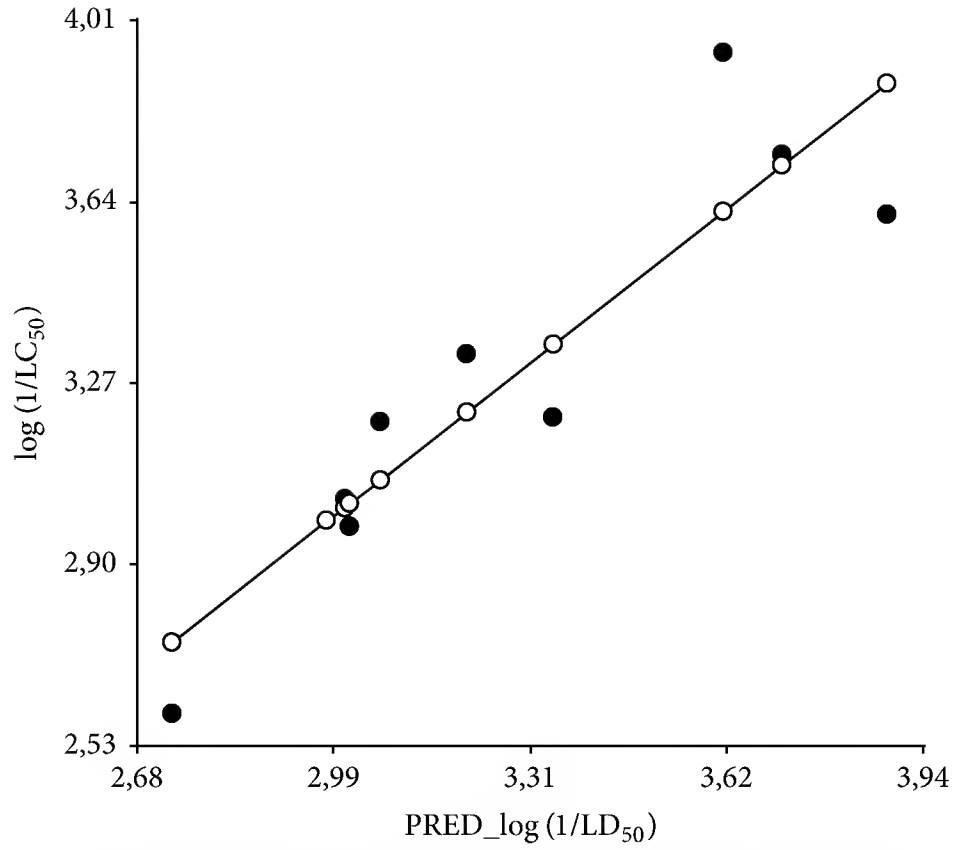

Figure 7: Multiple linear regression analysis (MLR) calculated to examine quantitative relationships between linear combinations of the dependent variable $\log(1/LD_{50})$ and the predictor variables (structure and molecular properties). Plot of calculated versus experimental $\log(1/LD_{50})$ of insecticidal activity of aldehyde and ketone compounds against cockroaches.

$$\log\left(\frac{1}{LD_{50}}\right) = 0.02\,(BP) + 0.01\,(ASA-) - 0.27\,(Pi\,E) - 0.02\,(Parachor) + 4.43, \quad (7)$$

$$N = 10;\ R^2 = 0.97;\ RMSPE = 9.1\%;\ P = 0.0006,$$

$$\log\left(\frac{1}{LD_{50}}\right) = -3{,}15\,(\log P) - 2.36\,(rings) - 0.09\,(MV) + 31.28, \quad (8)$$

$$N = 10;\ R^2 = 0.89;\ RMSPE = 6.3\%;\ P = 0.0029.$$

The plot of calculated versus experimental $\log(1/LD_{50})$ is shown in Figures 6, 7, and 8. The regression model (in (6)) suggests that the insecticidal activity of the alcohol and phenolic compounds increases with a rise in the solvent-accessible surface area for all hydrophobic atoms (ASA H). In contrast, in (8), this increases for the hydrocarbon compounds with a rise in the logarithm of the octanol/water partition coefficient ($\log P$).

In (7), the Pi energy (Pi E) of the ketone and aldehyde terpenes influences the insecticidal activity. The total Pi energy was calculated by the Huckel method and revealed a dissimilar charge distribution, which could result in a noncovalent interaction between aromatic amino acids of the proteins (e.g., AChE) and the organic compounds.

There has been much speculation about the structural relationship of terpenes and their bioactivity against cockroaches. Tortora et al. [61] argue that the degree of saturation of a compound may affect the detoxification because of which unsaturated terpenes may not be ordered in compact form, as the case of the saturated compounds, and consequently be more easily degraded. Steric aspects may also increase the solubility in the circulatory system of insects, allowing a more rapid excretion [42]. In fact, $\log P$ values of different terpenes have been interpreted in the light of their bioactivity in different ways. Matsumura [62] and Yu [47] found that high values of $\log P$ could enhance cuticular penetration and therefore favor insecticidal activity. However, monoterpene hydrocarbons with high $\log P$ values are less bioactive than those with lower ones [42]. Moreover, cuticles with a high content of lipid compounds can act as a trap for lipophilic substances and thus inhibit their traffic to the target site [47], implying that a positive relationship is not always found between lipophilicity and insecticidal activity [46, 48].

5. Triatominae Bugs (Vector of Chagas Disease)

Chagas disease is among the most important parasitic diseases of the southern region of America. It is caused by a protozoan parasite (*Trypanosoma cruzi*, Kinetoplastida, Trypanosomatidae) and can result at first in cardiological dysfunction and even lead to the patient's death [63]. The main vectors of the protozoan parasite are the hematophagous bugs *Rhodnius prolixus* (Stål, 1859), *Rhodnius neglectus* (Lent, 1954), and *Triatoma infestans* (Klug, 1834) (Hemiptera, Reduviidae, and Triatominae) [64, 65], which live in the inside and the surrounding areas of rural households. In Argentina, Bolivia, and Peru, the disease has been controlled since 1950 through elimination of the vector using synthetic insecticides [66]. Health agencies have used different strategies for the

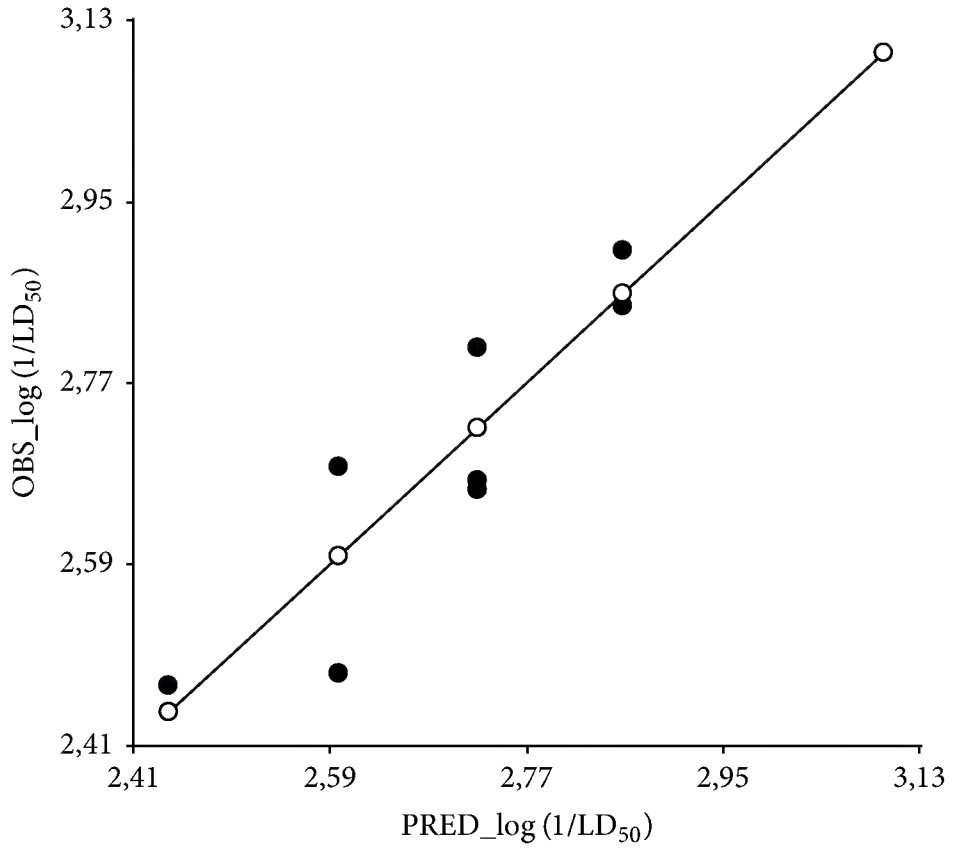

Figure 8: Multiple linear regression analysis (MLR) calculated to examine quantitative relationships between linear combinations of the dependent variable $\log(1/LD_{50})$ and the predictor variables (structure and molecular properties). Plot of calculated versus experimental $\log(1/LD_{50})$ of insecticidal activity of hydrocarbon compounds against cockroaches.

application of synthetic insecticides in rural households, with the most common method being the spraying technique, but painted surfaces with synthetic pesticides have also shown encouraging results [67, 68]. However, there are recent studies indicating the development of resistance by *T. infestans* [4, 69, 70]. An alternative to synthetic insecticides is the use of aliphatic alcohols, which have been demonstrated to help control mosquitoes and lice. When synthetic pyrethroids are used together with alcohols, synergistic effects have been observed, and it has been speculated that the alcohol may be affecting the cuticle of the exoskeleton of the insects and thereby facilitating the entry of the insecticides [71]. The cuticular surface is covered by a thin layer of lipids (mainly hydrocarbons, wax esters, and fatty alcohols) and with free or esterified fatty acids. These lipids play a major role in preventing lethal desiccation by altering the absorption of chemicals and microorganism penetration, and they also participate in chemical communication events [72–74]. Some fatty acids, by being part of the epicuticular waxes in *T. infestans*, have a strong action of regrouping (e.g., hexacosanoic acid) [75].

Essential oils are an alternative to synthetic pesticides for controlling blood sucking bugs [43, 76–78], with *Hedeoma mandoniana and Minthostachys andina* having been used as insecticides on the bugs *Rhodnius neglectus* and *Triatoma infestans*, and topical application revealing an insecticidal activity of 33% and 50% for *H. mandoniana* and *M. andina,* respectively. However, the evaluation of these essential oils as a fumigant produced different results, with *H. mandoniana* oil combined with 25.5% menthone and 33% isomenthone showing a mortality between 30 and 50% for the bugs, while *M. andina* with pulegone as the main component (44.6%) had no effect. Aliphatic alcohols appear to affect the cuticle of insects, thereby favoring the insecticidal action of some synthetic compounds. In addition, terpenes also affect components of the epicuticular waxes of insects, which could be a mechanism of pesticidal activity [79]. Repellent and ovicidal activities were also observed for different extracts from *Schinus molle* [80], and *Eucalyptus urograndis* essential oil had high insecticidal and repellent activities for *R. neglectus* nymphs [76, 77].

DEET (N,N-diethyl-3-methylbenzamide) is a synthetic compound that is used as repellent against mosquitoes and other Diptera, with the repellency of DEET on the bug vectors of Chagas disease having been tested by other authors [78, 81, 82]. As the antennae olfactory receptors of mosquitoes and bugs have been postulated as a possible site of action of DEET [82, 83], essential oil components could also have the same target.

We performed a quantitative analysis of the structure-activity relationship based on the work of Laurent et al. [43]. The oxygenated compounds menthol and alpha- and beta-thujone were omitted in the QSAR analysis because their larvicidal toxicity values were not determined in the cited reference, at the evaluated concentrations. These authors provided information about the menthol and thujone larvicidal toxicities as >to 2 mg/cm^2. The inaccuracy of this determination induced a decrease of model fit. The calculated molecular descriptors used in this analysis are shown in Table 6, and the plot of calculated versus experimental $\log(1/LD_{50})$ is shown in Figure 9. The model obtained using a contact toxicity bioassay of terpenes on nymphs is given by the following expression:

$$\log\left(\frac{1}{LD_{50}}\right) = 3.24\,(\mathrm{ASA}) + 0.0049\,(\mathrm{BP}) - 0.05\,(\mathrm{ST}) - 3.25\,(\mathrm{ASA}-) - 3.24\,(\mathrm{ASA}+) + 4.65, \quad (9)$$

$$N = 8;\ R^2 = 0.99;\ \mathrm{RMSPE} = 7.8\%;\ P = 0.0006.$$

This regression model (in (9)) suggests that the insecticidal activity of the alcohol and ketone compounds is influenced by the solvent-accessible surface areas.

Table 6: Calculated molecular descriptors[a] for compounds used in QSAR analysis of the insecticidal activity on triatominae nymphs.

Compounds[b]	ST	ASA	ASA+	ASA−	BP
Menthone	27.2	346.6	262.6	84.1	209
Isomenthol	29.7	293	228.6	64.3	218
Piperitone	28.9	335.2	244.8	90.4	234
Linalool	28.2	378.4	288.1	90.2	199
Pulegone	29.5	347	249.8	97.2	221
1,8-Cineole	32.4	307.7	242.4	65.3	176
Trans-anethole	31.8	389.6	299.6	89.8	237.5
Nerolidol	29.6	493	381.1	111.9	276

[a]The descriptors were calculated by ChemAxon and ChemSpider software.
[b]The data were obtained by Laurent et al. [43].

6. Potential Targets: Mode of Action of Essential Oil Components

Many insects have developed resistance to several classes of insecticides, which has created the need to search for and/or develop new effective pesticides to control them at any stage (egg, larvae, or adults). Resistance to insecticides may be defined as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species" [84]. Although the sites of action of synthetic pesticides are the most diverse, like action on the nervous system (AChE, gamma-aminobutyrate (Gaba), octopaminergic receptor, and sodium channel modulators), effects on the cuticle, growth regulators, and so forth [84], little is known about the sites of action of terpenes, despite pesticidal activity having been extensively studied.

The detoxification of aromatic structures is relatively more difficult than aliphatic ones and involves a series of biochemical processes that have a greater complexity. Thus, essential oils containing compounds with benzene-type structures are not easy for the insect to metabolize and detoxify, because they are more toxic than aliphatic compounds [47].

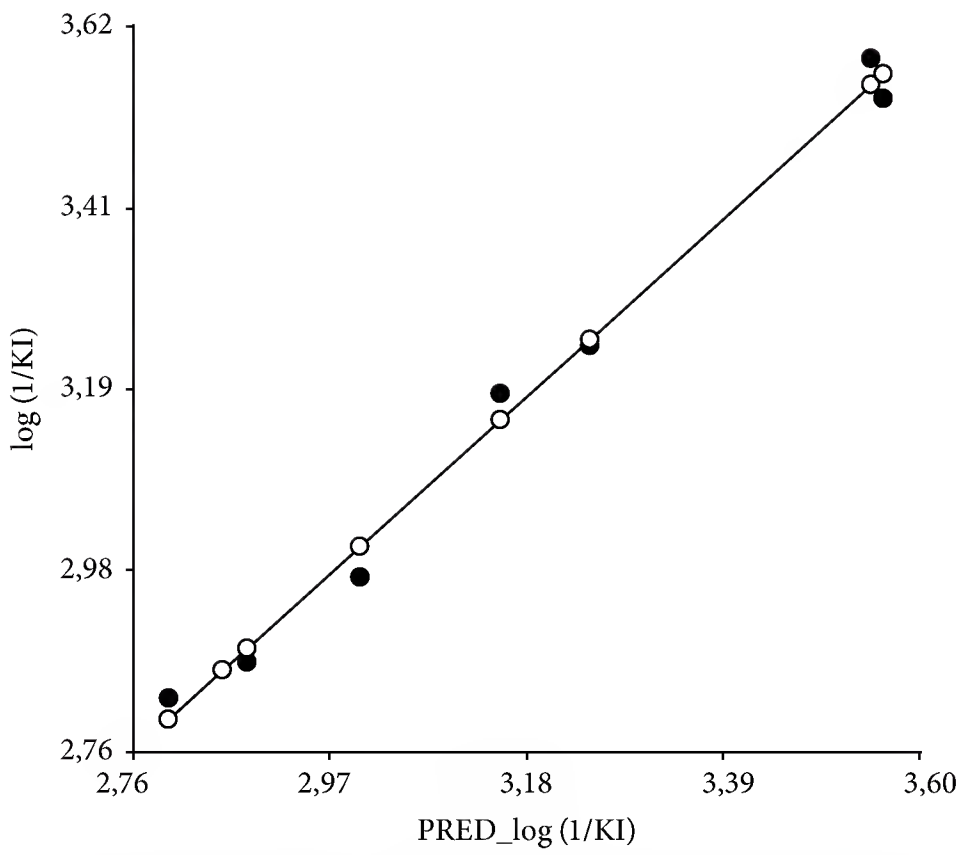

FIGURE 9: Multiple linear regression analysis (MLR) calculated to examine quantitative relationships between linear combinations of the dependent variable $\log(1/LD_{50})$ and the predictor variables (structure and molecular properties). Plot of calculated versus experimental $\log(1/LD_{50})$ of insecticidal activity against Triatominae bugs (vector of Chagas disease).

6.1. Acetylcholinesterase. Cholinesterases can be classified according to their substrate specificity as acetyl (AChE) or butyrylcholinesterase (BChE, E.C. 3.1.1.8). Inhibiting the AChE generates the accumulation of the neurotransmitter acetylcholine in neuronal synapses, which creates a state of permanent stimulation and results in a general lack of coordination in the neuromuscular system and subsequent death. Thus, the control of the activity of this enzyme is very important in determining the insecticidal capacity of natural or synthetic chemicals, with AChE consequently being the primary target of organophosphorus pesticides and carbamates [85, 86] as well as of some essential oils and their components [38, 49, 87, 88]. However, the AChE in resistant insects is able to change its active site or create new forms of the enzyme, in response to selective pressure after several years of the application of synthetic pesticides [86, 89–98].

Previous research has shown that essential oils from different herbs or their components can inhibit the activity of AChE. However, some of these studies employed AChE from electric eel, horse serum, or bovine erythrocytes [36, 87, 93, 94, 99–104], with future analysis of these results being difficult as the evaluation of the inhibitory effect on AChE enzyme was not performed in insects [32, 37, 93, 94, 99, 100, 104].

It has been suggested that insects may have only AChE with mixed properties of both vertebrate AChE and BChE [105]. Nevertheless, other studies have hypothesized that aphids, thrips, and probably some insects might have both AChE and BChE [95, 105]. In relation to this, an analysis of substrate hydrolysis at different concentrations revealed that insect cholinesterase displays activation at low substrate concentrations similar to that of the vertebrate BChE but demonstrated inhibition at high substrate concentrations similar to the vertebrate AChE [89]. Moreover, Keane and Ryan [96] reported that the inhibitory activity of monoterpenes against electric eel AChE is similar to the effect of inhibiting insect AChE.

Research on the insect AChE activity has involved extracts from *Musca domestica, Pieris brassicae, Galleria mellonella, Manduca sexta, Aphis citricola, Drosophila melanogaster* Meigen, *Apis mellifera, Blattella germanica, Pediculus humanus,* and *Tenebrio molitor*, with few studies having used AChE from insects that had been isolated and purified, for example, *Musca domestica, Rhyzopertha dominica, Lygus hesperus, Galleria mellonella*, and *Leptinotarsa decemlineata* [96, 97].

The terpenes previously evaluated as inhibitors of AChE activity on electric eel or bovine erythrocytes were 1,8-cineole, α-pinene, β-pinene, eugenol, α-terpineol, terpinen-4-ol, camphor, linalool, borneol, borneol acetate, pulegone, citral, fenchone, fenchol, 2-carene, 3-carene, verbenol, verbenone, trans-myrtanol, and myrtenol [37, 94, 96, 99, 100]. Among these monoterpenes, 1,8-cineole was found to be the best inhibitor of AChE activity (IC_{50} values 0.015–0.05 mg/mL), and it has also been reported to show great AChE inhibitory activity in different insects [32, 37, 96]. The hydrocarbon monoterpenes α-pinene and 3-carene showed a higher AChE inhibitory activity than their isomers β-pinene and 2-carene [37, 94, 99, 100], with the oxygenated monoterpenes such as linalool, pulegone, borneol, citral, α-terpineol, terpinen-4-ol, verbenol, myrtenol, myrtanol, fenchone, and fenchol being the natural products most commonly used to determine AChE inhibition. Moreover, these oxygenated compounds had less inhibitory effect on AChE than hydrocarbon terpenes (between 12 and 39% at

concentrations equal to or greater than 1 mM) [32, 37, 94, 96, 99, 100]. Finally, Savelev et al. [99] showed that 1,8-cineole can synergize its AChE inhibitory activity when mixed with α-pinene, with similar results being obtained when it was mixed with the β-pinene isomer. In contrast, a mixture of 1,8-cineole with camphor had an antagonistic effect, but limonene did not inhibit the AChE activity of *R. dominica* [97].

6.2. Octopaminergic Sites. Octopamine is present in the nervous system of arthropods, including insects, and acts both as a neurotransmitter and as a neurohormone. In addition, some researchers believe that octopamine modulates the influence of nerve-muscle interaction. Considering pharmacological criteria, octopamine exerts its effects through the octopamine-1 and octopamine-2 receptors [51, 97, 98], with its effect present throughout their union with G-protein-coupled receptors.

As the octopamine receptor binding is absent in vertebrates, it could be useful as a target for specific insecticide designs [87, 106]. However, only a few studies have shown octopamine receptor interaction with monoterpenes. Although the modulation of octopamine receptor by phenylpropenes or terpenes could be an indicator of the insecticidal activity of these natural compounds, conflicting results have been obtained concerning the interaction between the benzene and octopamine receptors. While thymol had no effect, its isomer carvacrol acted as an agonist at a concentration of 2 nmol/mL. The compounds p-cymene, eugenol, trans-anethole, vanillin, and isoeugenol were found to be antagonists of octopamine receptor [51], whereas in another study, from the electrophysiology point of view, eugenol did not show any antagonist activity [107]. Hence, there is no agreement about whether insecticidal activity of eugenol is directly related to its interaction with the octopamine receptor [51, 107, 108]. Chlordimeform, methomyl, permethrin, chlorfluazuron, malathion, trichlorfon, and some oxazolidine had agonistic effects, and eugenol, cinnamic alcohol, and phenyl methyl alcohol produced an increase in octopamine production in the German cockroach [109]. However, in contrast, limonene had no effect on the octopamine receptor of the American cockroach [51] or *R. dominica* [98].

Gross et al. [110], in a ligand-independent system, evaluated aliphatic and aromatic terpenes that acted as inverse agonists. One of the compounds tested, carvacrol, interacted with the octopamine receptor and altered the conformation and increased the affinity for the endogenous G-protein. Moreover, the QSAR models showed that electronic properties are the most important for the interaction of monoterpenoid with this receptor.

6.3. Gamma-Aminobutyrate (GABA). In insects, although inhibitory transmission relies mainly on three types of chloride-selective ligand-gated ion channels, namely, GABA-, glutamate-, and histamine-gated ionotropic receptors [111, 112], gamma-aminobutyrate (GABA) is a main inhibitory neurotransmitter in both vertebrates and invertebrates. Pharmacologically, the GABA receptor-chloride channel complex in insects is different from those present in mammals, as GABA receptors of insects have a regulatory function not only in the central nervous system, but also in the peripheral one [111, 113]. The terpenoids present in the essential oil of star anise (*Illicium* sp.) were found to show antagonist activity on the GABA receptor of *Musca domestica*, with these components also having structural features that permit a differential action between the GABA receptors of insects and mammals [113].

Carvacrol, pulegone, and thymol at concentrations between 500 mM and 1 mM are positive allosteric GABA receptors of the American cockroach. However, α-terpineol and linalool had no effect [54]. Waliwitiya et al. [114] reported in *Phaenicia sericata* that thymol interacts with the flight system and the central nervous system through the GABA complex. In addition, Priestley et al. [115] demonstrated the same result for *D. melanogaster*, with carvacrol and thymol being positive allosteric modulators of the GABA receptor of insects. Thus, the phenol group is an important part of the structure of terpene and exerts a positive modulation of the insect GABA receptor. Besides, the GABA receptor is more related to knocking down the insect than to insecticidal activity [53].

6.4. Tyramine Receptor. Tyramine is a biogenic amine that is biosynthetically and functionally related to octopamine, with this amine mediating the intracellular changes and producing second messengers such as cAMP and/or Ca^{2+}. Although tyramine is considered as a neurotransmitter, it is pharmacologically different from octopamine [116]. Enan [116] reported insecticidal activity of thymol, carvacrol, α-terpineol, p-cymene, and carvone on *D. melanogaster,* with the data indicating that thymol and carvacrol interact with the tyramine receptor without any significant differences and that the presence of a hydroxyl substituent on the benzene ring is critical for good insecticidal activity.

6.5. Growth Regulators. The use of natural compounds at sublethal concentrations can control insect pest populations by affecting growth and reproduction [117]. Terpenes can cause deformation of adult insect emergence or the lack of eggs, similar to growth-regulating hormones [118] such as juvenile hormone. Among the more active terpenes, geraniol causes a high rate of deformities in adults [119]. While the egg stage was found to be the most resistant, the larva was the most sensitive [120–122]. Oxide piperitenone retarded the reproduction of the malaria vector *Anopheles stephensi.* Moreover, oxide piperitenone also completely inhibited egg hatching at a dose of 750 μg/mL in an ovicidal assay, as well as inhibited oviposition, but no changes were observed in adult development [123]. When *Acanthoscelides obtectus* larvae and pupae were exposed to sublethal concentrations of lavender, rosemary, and *Eucalyptus* essential oil vapor, the results revealed an increase in the development times of larvae and pupae along with a reduction in the longevity and fecundity of the emerged female adults [124].

7. Discussion

The quantitative structure-activity relationship (QSAR) is a mathematical expression by which the chemical structure

is quantitatively correlated with well-known processes such as biological activity or chemical reactivity. The biological activity of a chemical compound depends on the ability to reach the specific target molecule and its subsequent capacity to interact with it ([125] and references therein). Hence, the three major types of interactions that the modeller must deal with are the hydrophobic, electronic, and steric ones [126]. For the different insects reported in the present study, the chemical characteristics represent a compound's ability to reach the target site, with the descriptors that mainly explained the insecticidal activities being BP, VP, and log P.

As the BP and VP descriptors represent the volatility of the compounds, it could be expected that these properties predict a higher activity in the less volatile compounds. In contrast, the opposite effect would be expected in the fumigant toxicity tests. The log P descriptor represents the lipophilicity of the compounds, which determines their ability to penetrate into the plasma membranes. In the obtained models, this descriptor only appeared in (8) (hydrocarbon compounds), indicating that an increase in lipophilicity leads to a decrease in activity. This is in agreement with Jang et al. [42], who suggested that the hydrocarbon monoterpenes with high log P values are less bioactive than those with lower log P, because they can be accumulated in the cuticle of insects and thus inhibit their traffic to the target site [47].

Other descriptors that largely contributed in the obtained models were MV and the number of rings. These structural characteristics are related to the steric aspect of the insecticidal activity of the compounds. Our obtained models revealed a higher activity of compounds with fewer rings and lower molar volume, which might indicate the importance of steric aspect in the interaction of molecules with the active sites of targets. This is in agreement with Rice and Coats [17], who suggested that monocyclic terpenes are more toxic than bicyclic on the German cockroach. The ASA descriptors in (4), (5), (6), and (7) indicate the importance of the interaction of the compounds with their target or vehicle, which is consistent with Lee et al. [52] and Jang et al. [42], who suggested the importance of interaction with water molecules on the surface of the insect respiratory system (tracheoles) and the bioactivity of these compounds. Finally, chemical descriptors such as RI, ST, and parachor demonstrate the importance of the interaction of monoterpenes with other molecules.

The results of several studies have suggested that insecticidal activity depends on several factors such as dose, species assayed, toxicity methods, and target [2, 44, 50, 127]. However, due to discrepancies in the methodologies used by different authors, comparison of the insecticidal effects for the evaluated compounds is difficult using these investigations. In addition, several authors evaluated the bioactivity of compounds with diverse chemical structures, which makes it difficult to attribute their activities to a specific chemical property. We think that a joint effort should be made to standardize the methodology used and a standard compound (universal) could be added that would allow a comparison of the activity using the results of different authors.

In conclusion, the present work demonstrated that molecular properties or descriptors are important for explaining the bioactivity of terpene compounds on insects. The resulting models employed steric, electronic, and transport features to predict the bioactivity, with the results suggesting that the activity of these compounds depends on their ability to reach the targets, and their capability of interacting with them. In addition, the mathematical expression obtained by the QSAR analysis can be used to predict the insecticidal activities of structurally related molecules. Our findings may provide an important contribution in the search for new compounds with insecticidal activity. In addition, the development of natural insecticides has some advantages; for example, they are naturally occurring, they have low toxicity against nontarget organism, they do not persist in the environment, they have the capability to not generate resistant populations, and they have nonrestricted use [128, 129]. Based on these properties and despite the high cost of natural products, the benefits related with their use could be an alternative to synthetic pesticide.

Abbreviations

ASA: Solvent-accessible surface area calculated using the radius of the solvent (1.4 Å for water molecule)
ASA+: Solvent-accessible surface area of all atoms with positive partial charge (strictly greater than 0)
ASA−: Solvent-accessible surface area of all atoms with negative partial charge (strictly less than 0)
ASA H: Solvent-accessible surface area of all hydrophobic ($|qi| < 0.125$) atoms ($|qi|$ is the absolute value of the partial charge of the atom)
ASA P: Solvent-accessible surface area of all polar ($|qi| > 0.125$) atoms ($|qi|$ is the absolute value of the partial charge of the atom)
BP: Boiling point
DP3D: Plane deviation 3D
FW: Formula weight
HBA: Hydrogen bond acceptor
HBD: Hydrogen bond donor
log P: Logarithm of the octanol-water partition coefficient
MR: Molar refractivity
MV: Molar volume
OE: Orbital electronegativity
P: Polarizability
Pi E: Pi energy
pka: Logarithm of dissociation constant
PSA: Polar surface area
RI: Refractive index
ST: Surface tension
VE: Vaporization enthalpy
VP: Vapor pressure.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Research Article

Perch Selection by Three Cooccurring Species of *Celithemis* (Odonata: Libellulidae): Testing for a Competitive Hierarchy among Similar Species

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In many communities of perching dragonflies (Odonata: Libellulidae), a size-dependent competitive hierarchy creates a positive relationship between male body size and perch height. We tested for this pattern among three similar-sized species: *Celithemis elisa*, *C. fasciata*, and *C. ornata*. Males were caught and photographed from May to July 2015 at Ashmore Heritage Preserve, Greenville County, SC, USA, and perch heights and perch distance to open water were measured. Five indices of body size were measured with ImageJ software: abdomen length, forewing length, hindwing length, area of forewing, and area of hindwing. *Celithemis fasciata* was significantly larger than the other two species for all five anatomical characters and used perches that were significantly taller and closer to open water than the other species, though these differences changed over the summer. Aggressive interactions between and within species were tallied and compared to expected distributions based on mean relative abundances derived from hourly abundance counts. Patterns of interspecific aggression were also consistent with a size-dependent hierarchy: the large *C. fasciata* was attacked less frequently, and the small *C. ornata* more frequently, than predicted by their relative abundances. We conclude that even small differences in body size may contribute to niche partitioning in perch selection.

1. Introduction

In general, large size confers a competitive advantage to animals engaged in physical combat [1–3]. As a consequence of greater strength and the application of greater force, larger animals are more likely to win contests for mates, territories, and other resources [2, 3]. Many dragonfly (order: Odonata) species are highly aggressive and territorial [4]; males perch on vegetation along the periphery of a water body and defend small territories around oviposition sites, driving off other males and attempting to mate with passing females. Appropriate perch selection has important benefits for reproductive success [5–8], and size-dependent hierarchies occur in both intraspecific and interspecific patterns of territory acquisition and perch selection.

In *Pachydiplax longipennis*, for example, larger males are typically the aggressor in intraspecific interactions, forcing smaller males to disperse [9]. In several other species, males holding territories either are larger than "satellite" males without territories (suggesting a size advantage for acquiring territories) or win more intraspecific contests [10–14]. However, because body size differences within a species are often small, other factors like age, fat reserves, or sexual ornamentation can be more important than size to the outcome of a particular competitive battle [15]. Residency itself can provide a competitive advantage; territory owners are more likely to win contests than challengers, perhaps because of the competitive edge that earned them the territory in the first place or their greater familiarity with the site [4, 16–20].

Competitive hierarchies also exist between species, particularly with respect to perch selection. Because perches are used to survey territories for food, mates, predators, and intruders, maximum visibility is probably a key component of perch quality [21]. Tall perches provide better visibility than short perches, particularly as the distance from open water increases and the view from short perches is obstructed by

intervening vegetation. As predicted, competitively dominant large species use tall perches and relegate progressively smaller species to progressively shorter perches [22–27]. *Perithemis tenera*, a small species (20 mm), uses short perches (<20 cm) but maximizes visibility by selecting perches that are beyond the shoreline in open water [21].

There are exceptions to these patterns, however, usually among closely related species that are similar in size. For example, *Sympetrum flaveolum* is slightly larger than *Sympetrum sanguineum* but uses shorter perches, probably as a consequence of exploiting earlier successional habitats with shorter vegetation [28]. In competitive interactions among closely related similar-sized species, the same factors that affect intraspecific competition may become important, like age, fat reserves, or residency [15]. Residency is a stronger predictor of competitive success than size in competitive interactions among five species of *Erythrodiplax* [29]. When size differences between species are small or inconsistent, size-dependent differences in perch height alone may not be enough to promote coexistence; selection may favor resource partitioning along additional niche dimensions like seasonality [30], diel period [31, 32], habitat [28, 33], or another characteristic of the perch like distance from shore [21].

The genus *Celithemis* provides an ideal model system for examining these relationships. All of the eight species of *Celithemis* occur in the Eastern United States [34], with a narrow size range from *Celithemis amanda* (22 mm body length) to *Celithemis eponina* (38 mm) [35]. Most ponds and marshes in the region will harbor at least 2-3 species, particularly in southeastern states where as many as five *Celithemis* species can co-occur [36]. Lastly, *Celithemis elisa*, *C. fasciata*, and *C. ornata* are territorial; they exhibit both "site attachment" and "agonistic defense" [37]. This study had three objectives: (i) compare the body sizes of *Celithemis elisa*, *C. fasciata*, and *C. ornata* males and determine if patterns of perch selection (based on perch height and the distance of perches from open water) correlate with differences in body size; (ii) describe patterns of diel and seasonal activity to determine whether these species partition resources temporally; and (iii) describe patterns of intra- and interspecific aggression among these species to determine whether the patterns of spatial and temporal perch selection are consistent with a size-dependent competitive hierarchy.

2. Materials and Methods

2.1. Study Site. This research was conducted at Lake Wattacoo at the Ashmore Heritage Preserve, Greenville County, SC, USA (latitude: 35° 5′ 6.83″ N, longitude: 82° 34′ 43.64″ W, elevation 347 m). The lake is a 2.2 ha impoundment on the 455 ha preserve, directly below the southeastern escarpment of the Blue Ridge Mountains [38]. The lake is bordered on two sides by mixed oak-pine woodlands. The study was conducted along 200 m of treeless lakeshore on the earthen dam. The steep slope of the dam limits rooted macrophytes to a zone of reeds extending 1-2 m from the bank. These reeds and the shoreline vegetation were used as perches by territorial male dragonflies. *Celithemis elisa* (Hagen), *Celithemis fasciata* Kirby, and *Celithemis ornata* (Rambur) are the most abundant libellulids at the site, constituting 85% of the libellulid individuals in a 2014 survey [39]. The most common species in the region, *Pachydiplax longipennis* (Burmeister), *Libellula incesta* Hagen, and *Erythemis simplicicollis* (Say), are present but uncommon at Wattacoo, so interactions among *Celithemis* species can be studied without the complicating effects of other species.

2.2. Sampling Protocol. Sampling was conducted from May to July 2015, 3-4 days/week, in 3–6-hour blocks of rainless conditions, between 1000 h and 1600 h. At the stroke of each hour, the 200 m dam was walked twice ("out" and "back" transects); the number of individuals of each species seen on each transect was counted, and the counts were averaged to compute an hourly abundance for each species. Between these hourly counts, we randomly shifted our activity between collecting males for body size measurements, measuring perch heights of territorial males, and observing aggressive interactions. These activities were done at different times in different areas on different males, so that our swinging nets and measuring perches would not disturb observations. Males were collected by aerial net, numbered on their wing with a Sharpie© marker for identification (and to prevent resampling), photographed with a ruler for scale, and released. Males were collected at the shoreline and along the bank, so both territory holders and satellite males were collected and measured. For territorial perches used by males, the vertical height and horizontal distance to open water were measured. Perches were considered "territorial" if they were along the shoreline (not on the bank) or on emergent vegetation. Aggressive interactions were scored by observing a target area (approximately 5 m of shoreline that contained perched dragonflies) for 15–30 minutes and recording as many interactions as possible. An "attack" was scored when a perched dragonfly was attacked by another dragonfly. A "sortie" was scored when a perched dragonfly left the perch to charge a passing dragonfly and return to its perch. A "chase" was scored when one dragonfly pursued another. The interactions can be rapid and dynamic; a perched dragonfly might be attacked, initiate a sortie against the attacker, and then be chased in an ensuing dogfight [40]. Each of these three interactions was scored separately, noting the species of the aggressor and target for each.

2.3. Testing the Relationship between Body Size and Perch Selection. Five attributes of body size were measured on each dragonfly photograph using ImageJ software [41]: length and area of forewing, length and area of hindwing, and abdomen length. Variations in these parameters between species were assessed and described with MANOVA, ANOVA, and Tukey mean comparison tests. Variations between species and across months in perch height and distance from perch to open water were assessed with two-way factorial ANOVA and Tukey mean comparison tests and compared with predictions based on a size-dependent competitive hierarchy.

2.4. Testing for Patterns of Temporal Partitioning. Variations between species, across diel period, and across months

Table 1: Summary of ANOVA describing the variation in five anatomical characters between male *Celithemis ornata*, *C. elisa*, and *C. fasciata* collected from May to July 2015 at Ashmore Heritage Trust Preserve, Greenville County, USA: length of forewing, length of hindwing, abdomen length, area of forewing, and area of hindwing.

Character	Species effect		Error		F	P
	df	MS	df	MS		
Forewing length	2	50.258	218	3.799	13.230	0.0001
Hindwing length	2	46.499	218	3.647	12.748	0.0001
Abdomen length	2	41.602	218	2.632	15.809	0.0001
Forewing area	2	6265.947	218	436.550	14.353	0.0001
Hindwing area	2	9789.665	218	636.533	15.380	0.0001

in mean hourly abundances were assessed with three-way factorial ANOVA and Tukey mean comparison tests.

2.5. Testing for a Size-Dependent Competitive Hierarchy. The observed frequencies with which each *Celithemis* species engaged the three species in each interaction were pooled across the entire sampling period. For each behavior (attack, sortie, and chase), we compared the frequencies at which each species acted aggressively towards each of the three species. To determine whether a species preferred or avoided acting aggressively towards another species, we compared these observed values with frequencies we would expect if there was no preference. If a species shows no preference or avoidance, it should engage target species at the same proportions as the species occur in the environment. We computed the proportional representation of each species in the environment by (i) calculating the mean hourly abundance values averaged over the entire sampling period; (ii) totaling these means; and (iii) computing the proportional representation of each species to this total. For each contrast, expected values were generated by multiplying the total number of observations for that contrast by these proportions. Observed and expected values were compared with chi-square goodness of fit tests. All statistical tests were conducted using SPSS software [42].

3. Results

3.1. Relationships between Body Size and Perch Selection. There were statistically significant differences among these three study species in the five attributes of body size, whether patterns are analyzed concurrently (MANOVA, Wilk's λ, $F = 8.173$, df = 10, 428, $P < 0.0001$) or in separate ANOVA (Table 1). For each of the five attributes, *C. fasciata* was significantly larger than the other two species (Figure 1). Although *C. elisa* was larger than *C. ornata* in four of the five variables (not abdomen length), none of these differences were statistically significant (Figure 1).

There were statistically significant differences between species for both perch height and the distance from the perch to open water ("species" effect, Tables 2(a) and 2(b)). Overall, *C. ornata* used perches that were significantly shorter than those used by the other two species, while *C. fasciata* used

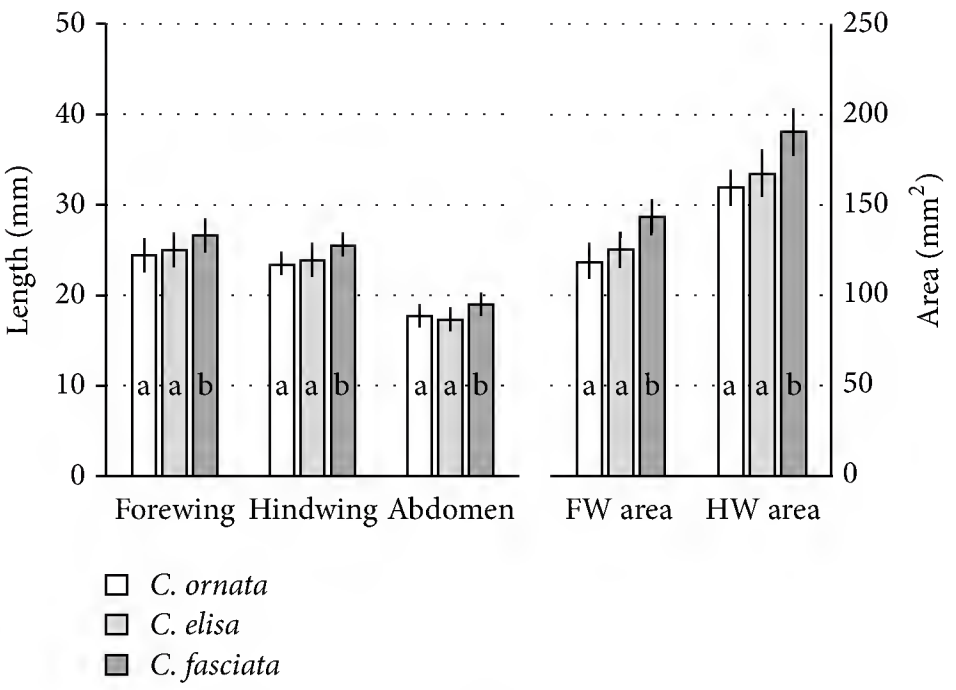

Figure 1: Mean comparisons of five body size indicators between males of *Celithemis ornata* ($N = 51$), *C. elisa* ($N = 139$), and *C. fasciata* ($N = 31$) collected at Ashmore Heritage Preserve, Greenville County, SC, USA, from May to July 2015. Mean (± SD) lengths of forewing, hindwing, and the abdomen and the area of forewing (FW area) and hindwing (HW area) were compared. For each attribute, species labeled with the same letter are not significantly different (Tukey mean comparison tests, $P = 0.05$).

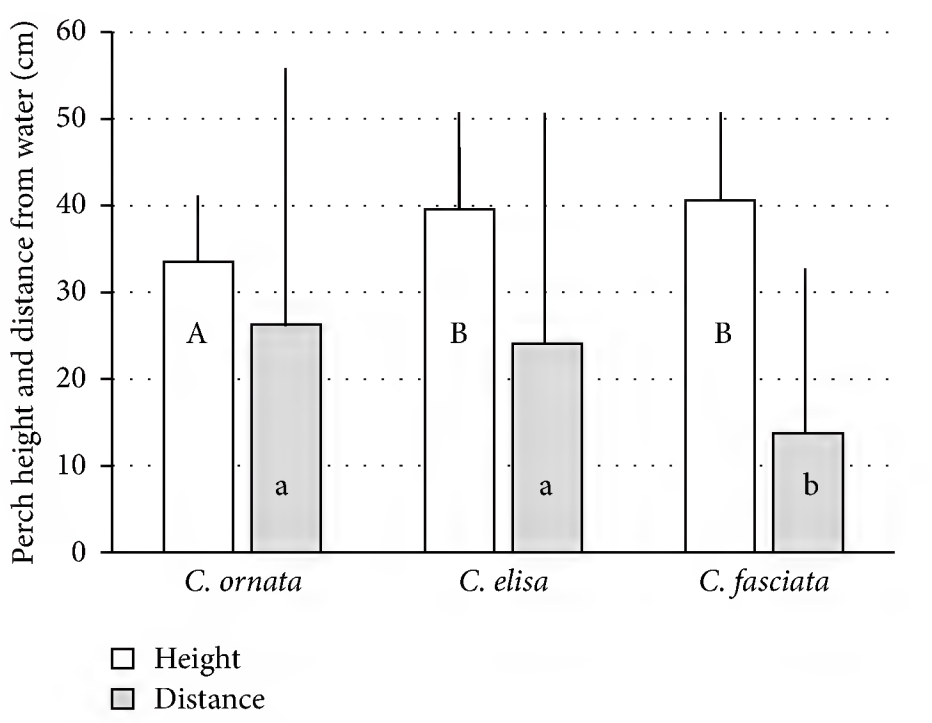

Figure 2: Mean (+ SD) perch height and distance from open water (cm) for perches used by male *Celithemis ornata* ($N = 278$), *C. elisa* ($N = 331$), and *C. fasciata* ($N = 207$) at Ashmore Heritage Preserve, Greenville County, SC, USA, from May to July 2015. For each variable, species labeled with the same letter are not significantly different (Tukey mean comparison tests, $P = 0.05$).

perches that were significantly closer to water (Figure 2). Mean perch height tended to increase over the summer while perch distance from the water tends to decrease ("month" effect, Tables 2(a) and 2(b)). These trends, however, were not consistent across species ("species × month" effect, Tables 2(a) and 2(b)). Mean perch height of *C. elisa* increased by 12 cm over the summer, which was twice the increase of the other two species (Figure 3(a)). Likewise, although *C. ornata* and *C. elisa* perched closer to the water as summer

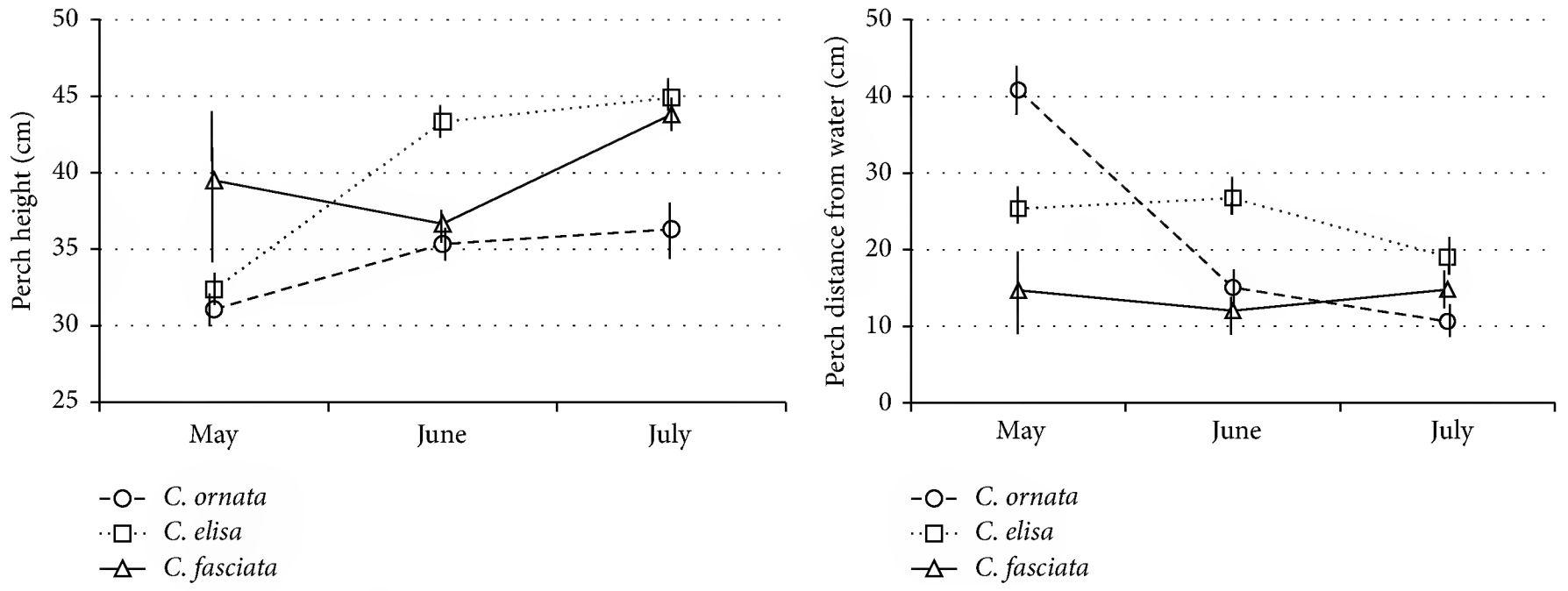

Figure 3: Monthly changes in (a) perch height (mean ± SE) and (b) perch distance from open water (mean ± SE) for *Celithemis ornata*, *C. elisa*, and *C. fasciata* at Ashmore Heritage Preserve, Greenville County, SC, USA.

Table 2: ANOVA describing the direct and interactive effects of species ("species") and sampling period ("month") on (a) perch height and (b) perch distance from open water, for males of *Celithemis ornata*, *C. elisa*, and *C. fasciata* at Ashmore Heritage Trust Preserve, Greenville County, USA.

(a)

Source	df	Type III SS	MS	*F*	*P*
Species	2	5475.492	2737.746	28.709	0.0001
Month	2	4684.503	2342.251	24.561	0.0001
Species × month	4	3613.844	903.461	9.474	0.0001
Error	807	76958.345	95.364		

(b)

Source	df	Type III SS	MS	*F*	*P*
Species	2	9028.425	4514.212	6.108	0.002
Month	2	12965.444	6482.722	8.772	0.0001
Species × month	4	29343.458	7335.865	9.926	0.0001
Error	807	596397.391	737.030		

progressed, *C. fasciata* tended to perch near the water throughout the sampling period (Figure 3(b)).

3.2. Patterns of Temporal Partitioning. There was a significant difference in the mean hourly abundances of these three species overall ("species" effect, Table 3); on average, *C. elisa* (mean ± 1 SE = 13.34 ± 0.65, $N = 178$) was significantly more abundant than *C. fasciata* (7.34 ± 0.63, $N = 178$) and *C. ornata* (6.56 ± 0.39, $N = 178$) which did not differ from one another (Tukey mean comparison test, $P = 0.05$). However, these patterns changed significantly over the course of the summer ("species × month" effect, Table 3). While *C. elisa* maintained a consistently high abundance throughout the summer, *C. ornata* abundance declined and *C. fasciata* abundance increased as summer progressed (Figure 4). There was also a significant change in abundance over the course of

Table 3: ANOVA describing the direct and interactive effects of species ("species"), diel period ("hour"), and sampling period ("month") on the mean abundance of *Celithemis ornata*, *C. elisa*, and *C. fasciata* at Ashmore Heritage Trust Preserve, Greenville County, USA, from May to July 2015.

Source	df	Type III SS	MS	*F*	*P*
Species	2	2722.832	1361.416	33.577	0.0001
Hour	6	2826.822	471.137	11.620	0.0001
Month	2	96.338	48.169	2.830	0.024
Species × hour	12	521.047	43.421	1.071	0.383
Species × month	4	3450.909	864.977	21.333	0.0001
Hour × month	12	689.849	57.487	1.418	0.154
Species × hour × month	24	433.684	18.070	0.446	0.990
Error	434	17596.866	40.546		

a day ("hour" effect, Table 3), but this pattern was consistent across the three species ("species × hour" effect, Table 3). For all species, abundance increased from 1000 h to 1300 h and then decreased towards 1600 h (Figure 5). These diurnal patterns did not change through the summer ("species × month × hour" effect, Table 3).

3.3. Agonistic Interactions and a Size-Dependent Competitive Hierarchy. A total of 1184 attacks, sorties, and chases between these species were tallied. All the three species were significantly more likely to "chase" conspecifics than heterospecifics (*C. elisa*: $\chi^2 = 175.28$, df = 2, $P < 0.001$; *C. fasciata*: $\chi^2 = 107.19$, df = 2, $P < 0.001$; *C. ornata*: $\chi^2 = 121.83$, df = 2, $P < 0.001$). With respect to initiating a "sortie" from a perch to investigate a passing dragonfly, *C. elisa* and *C. ornata* were significantly more aggressive towards conspecifics and less responsive to the other two species (*C. elisa*: $\chi^2 = 138.53$, df = 2, $P < 0.001$; *C. ornata*: $\chi^2 = 23.61$, df = 2, $P < 0.001$). *Celithemis fasciata* showed no preference, initiating investigative sorties against passing dragonflies at the same rates at which these

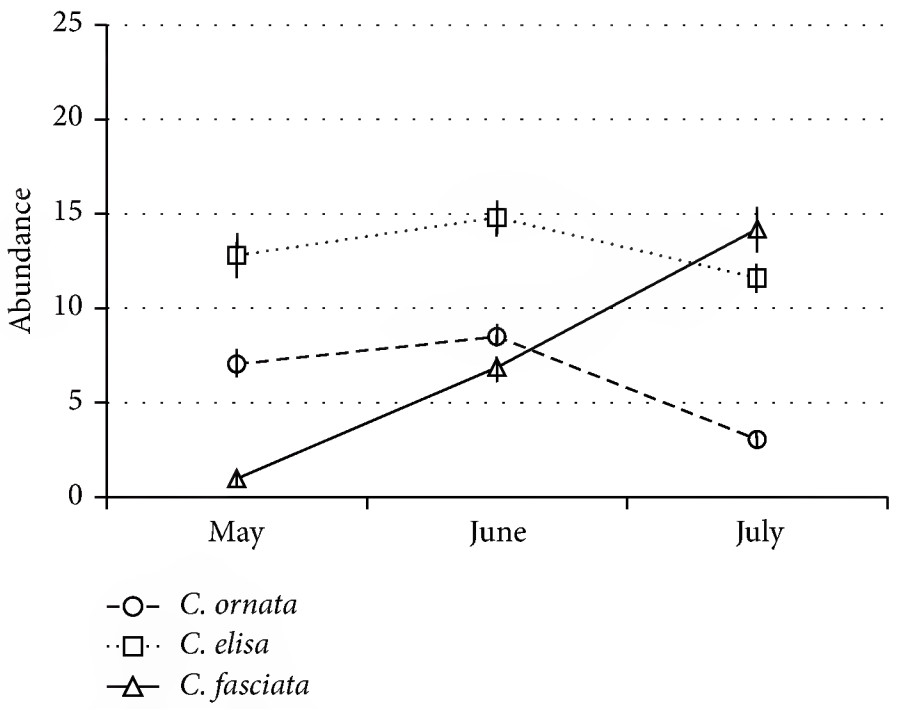

FIGURE 4: Monthly changes in the hourly abundance counts ($X \pm$SE), for *Celithemis ornata*, *C. elisa*, and *C. fasciata* at Ashmore Heritage Preserve, Greenville County, SC, USA, from May to July 2015.

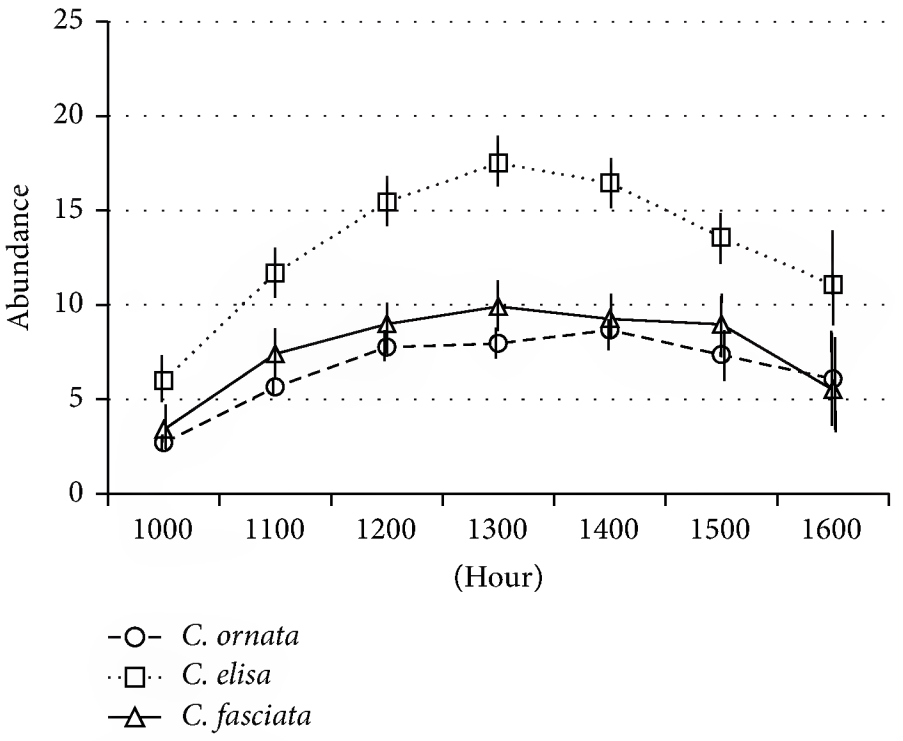

FIGURE 5: Diel changes in hourly abundance counts ($X \pm$ SE), averaged over the entire sampling period, for *Celithemis ornata*, *C. elisa*, and *C. fasciata* at Ashmore Heritage Preserve, Greenville County, SC, USA, from May to July 2015.

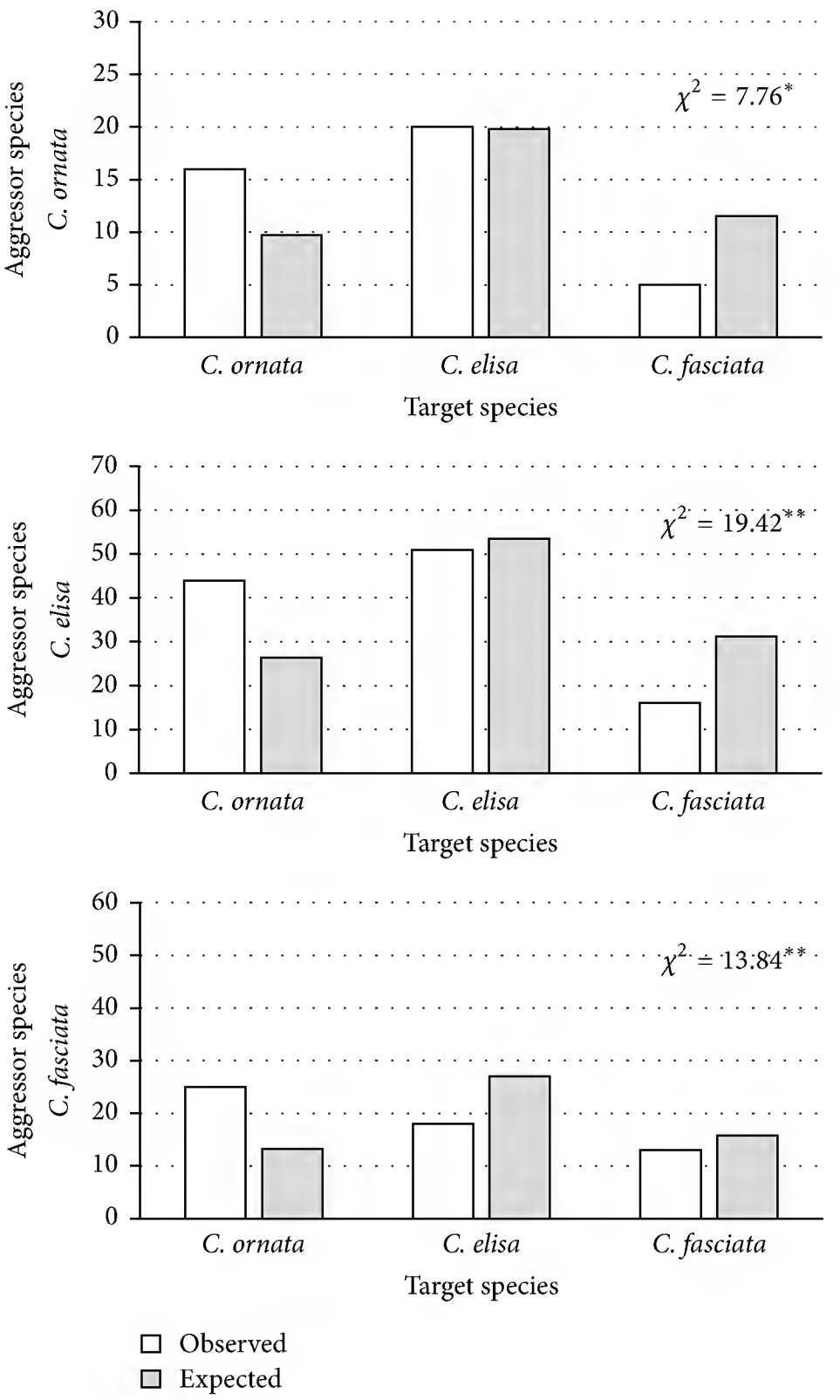

FIGURE 6: The frequency at which *Celithemis ornata*, *C. elisa*, and *C. fasciata* attacked perched individuals of these species, compared to expected frequencies based on relative mean hourly abundance counts averaged over the entire sampling period of May–July 2015 ($^*P < 0.05$; $^{**}P < 0.01$, chi-squared goodness of fit tests).

species occurred in the environment ($\chi^2 = 0.939$, df = 2, $P > 0.05$). When it came to attacking a perched individual, these preferences for engaging conspecifics changed: all the three species attacked *C. ornata* more frequently and *C. fasciata* less frequently than expected by their relative abundances (Figure 6).

4. Discussion

Although males of *Celithemis elisa*, *C. fasciata*, and *C. ornata* broadly overlap in size and perch use, a size-dependent competitive hierarchy influenced territorial perch selection. On average, *Celithemis fasciata* was significantly larger than the other two species in all five body size attributes, was avoided by both smaller species when occupying a territorial perch, and used perches that were either significantly taller or closer to open water than the two smaller species. In addition, although size differences between *C. ornata* and *C. elisa* were not statistically significant, the smaller *C. ornata* males used significantly shorter perches than *C. elisa*, perched farther from the water (though not significantly farther than *C. elisa*), and were attacked more frequently by all species than expected by their relative abundance. In short, although all differences were not statistically significant, there were consistent rank-order patterns in perch height, perch distance, and vulnerability to attack that correspond to rank-order differences in mean body size. These patterns are consistent with the size-dependent niche partitioning of perch selection documented in other dragonfly assemblages [22–27].

It is illustrative to compare the results of the three aggressive behaviors. All three species preferentially chased conspecifics over the other two species, and *C. elisa* and *C. ornata* preferentially initiated sorties against conspecifics. These behaviors are typical [4, 5, 15, 22–24, 29, 43] and adaptive for males that hold a territory [15]; by engaging conspecifics through a sortie or chase, they can identify mature females or drive off other male suitors. For perch/territory acquisition, however, a different pattern would be expected if there were a size-dependent competitive hierarchy. Under this hypothesis, territorial males of the largest, competitively dominant species should be avoided by attacking dragonflies; even conspecific aggressors are likely to lose a competitive battle to a territorial resident [4, 16–20]. In contrast, territorial males of the smallest, competitively subordinate species should be attacked disproportionately by all species, even by conspecifics that have a better chance against a small member of their own species than against an individual of a larger species. These were the patterns we observed: all the three species attacked the large *C. fasciata* less frequently and the small *C. ornata* more frequently than expected by their relative abundances.

An alternative hypothesis that explains differences in interspecific aggression is "mistaken identity," where morphologically similar species interact more frequently than less similar species because of imperfect species recognition cues [43–45]. Although similar in size, the *Celithemis* species in this study are rather different morphologically. *Celithemis ornata* has a reddish basal spot on the hind wings, *C. elisa* has reddish spots at the tip and nodus of each wing in addition to the reddish basal hindwing spot, and *C. fasciata* has wings that are heavily marked in black spots. If anything, it seems they have already experienced the character displacement predicted for closely related species to reduce interference competition for mates [46]. And again, although conspecifics were the focus of sorties and chases, there is a different pattern for attacking perched individuals. If mistaken identity was the cause of these interspecific behaviors, there should be the lowest error rate for attacking a stationary individual on a perch, with their wings outstretched and visible. But all species showed a nonrandom preference for attacking *C. ornata* and avoiding *C. fasciata*. These patterns are more consistent with size-dependent interspecific aggression for perch acquisition than mistaken identity of conspecifics.

Although gross patterns in perch selection support the hypothesis of a size-dependent hierarchy between these species, there was considerable variability in every metric. First, there was broad overlap in the size ranges of these species, such that large *C. ornata* were larger than small *C. elisa* and nearly equal in size to small *C. fasciata*. Likewise, all three species used perches between 10 and 90 cm in height, from the shoreline to open water. So, although these patterns hold when means are compared at the species level, individual body size may be a better predictor of competitive ability than species identity. There were also significant temporal changes in the characteristics of the perches used by each species. Mean perch height increased through the summer for all species, probably as a consequence of the growth of vegetation. However, mean perch height of *C. elisa* increased more dramatically than for the other two species. Indeed, in June, *C. elisa* had a significantly higher perch height than the larger *C. fasciata*. Patterns in the distance from open water of perches used by these species also changed. Although *C. ornata* used perches farthest from open water in May, this species perched closer to open water than the other two species in July. As summer progresses and vegetation grows, it becomes more difficult to see open water from short, distant perches. *C. ornata* males might shift to taller perches closer to open water to procure a mate, even if it means tolerating more harassment from larger, competitively superior species.

The presence of species-specific temporal patterns varied with temporal scale. As in *Sympetrum danae* and *S. pedemontanum* [32], there was no evidence of temporal partitioning by diel activity; all the three *Celithemis* species increased in abundance from 1000 h to 1300 h and then declined to 1600 h. There were, however, significant differences in abundance through the sampling period: *C. elisa* was abundant throughout, *C. ornata* is an early-season species that began to decline in July, and *C. fasciata* is a late-season species that rose dramatically in July. These trends are consistent with the flight dates listed by Beaton [30]. Although there were significant differences in seasonal abundance patterns, these species coexist through the majority of their flight seasons and, during this period, tend to partition perches spatially rather than temporally.

5. Conclusion

Body size is a critical variable that affects and responds to competitive interactions in ecological communities [1–3]. In many communities of perching dragonflies, species partition perches based on perch height [22–27]. As a consequence of a size-dependent competitive hierarchy, larger species use taller perches and relegate progressively smaller species to progressively shorter perches [25–27]. This study documents the same pattern on a smaller scale, among three *Celithemis* species that are very similar in size. In addition, larger species perched closer to open water, which also may represent competitive dominance for a high-quality resource. These patterns were weak, however, and changed over the summer, perhaps as other variables became more important than these slight differences in body size or as species responded to changes in the relative quality of perches.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Defensive Nymphs of the Woolly Aphid *Thoracaphis kashifolia* (Hemiptera) on the Oak *Quercus glauca*

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Aphid nymphs with enlarged fore- and mid-legs were found from woolly colonies of *Thoracaphis kashifolia* (Hormaphidinae, Nipponaphidini) on leaves of the evergreen *Quercus glauca* in Japan. It was shown that they grasped an introduced moth larva with their legs and some inserted their stylets deep into the body. These defenders were first-instar nymphs of the alate generation and were produced by aleyrodiform apterae from early September onward. There was a large variation in the size of their forelegs. First-instar nymphs (to be alates) produced early in the season had fore-femorotrochanters shorter than those produced later. The molting rate (the percentage of pharate individuals) of the latter was very low (less than 5% to zero), suggesting their semisterility. Although first-instar nymphs with various lengths of forelegs joined to attack moth larvae, these facts indicate that an incipient caste differentiation occurs within the first-instar nymphs of the alate generation.

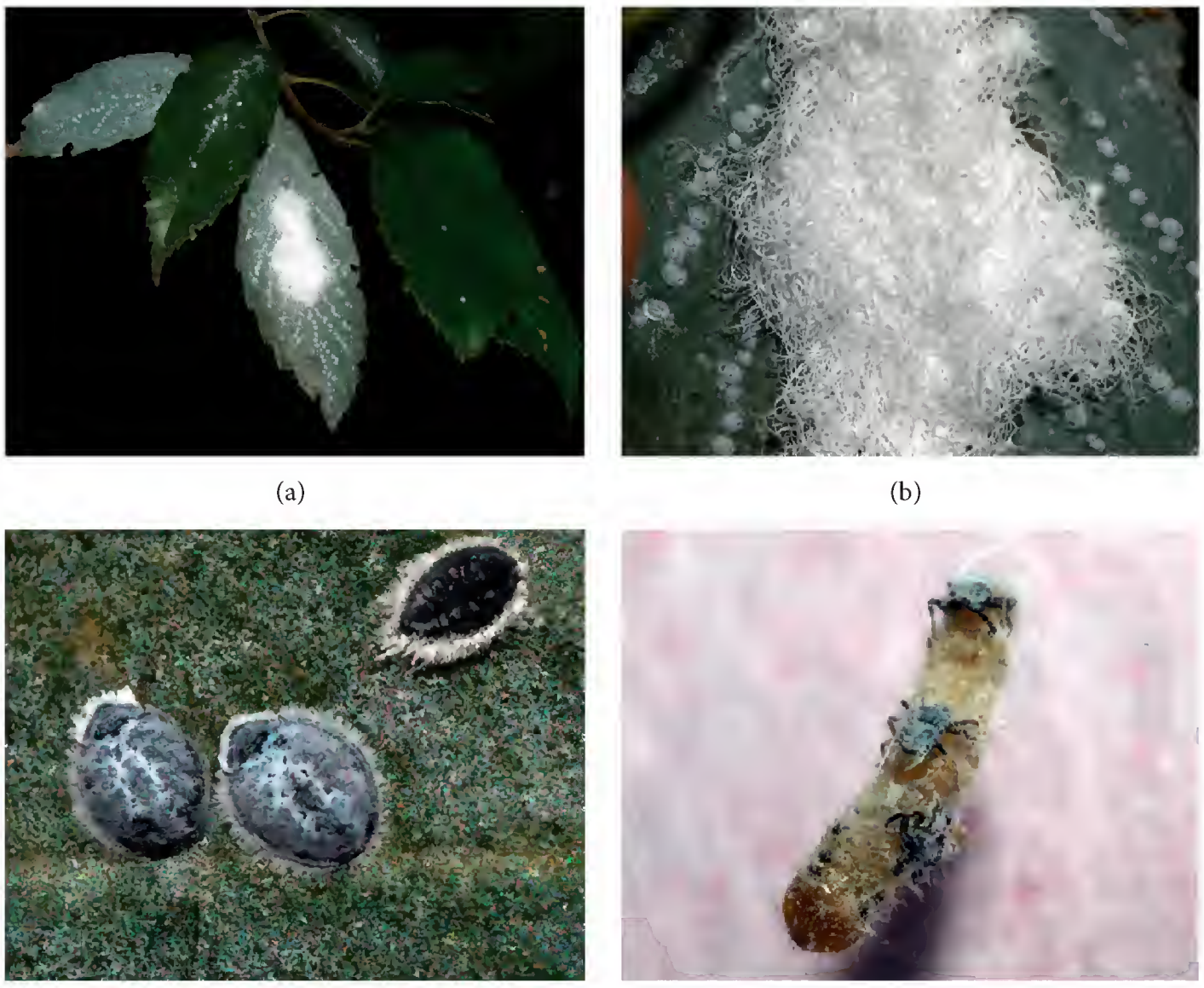
1. Introduction

Since the discovery of aphid soldiers in *Colophina clematis* [1], sterile or nonsterile defensive nymphs have been found in many species of two aphid subfamilies, Eriosomatinae and Hormaphidinae [2–5]. The former subfamily consists of three tribes, Eriosomatini, Pemphigini, and Fordini, and the latter also consists of three, Hormaphidini, Cerataphidini, and Nipponaphidini [6, 7]. These aphid species basically have a host-alternating life cycle; most of them induce galls on their primary host and form exposed colonies on their secondary host. Defensive individuals (usually first- or second-instar nymphs [2] but at times fourth-instar nymphs [8] or apterous adults [9, 10]) have been recorded from the gall or the primary-host generations of all six tribes [9–17], and from the secondary-host generations of Eriosomatini [1], Cerataphidini [15, 18–21], and Hormaphidini [22]. Although exules of *Paracletus cimiciformis* (Fordini) have recently been shown to suck on ant larva hemolymph [23], defensive behavior on the secondary host has been unknown from the remaining three tribes to date.

In the course of studying the life cycle of the aphid *Thoracaphis kashifolia* (Nipponaphidini) in Japan, we noticed soldier-like first-instar nymphs with thickened fore- and mid-legs in its woolly colonies on leaves of the evergreen oak *Quercus glauca*. Having introduced lepidopteran larvae onto the colonies, we succeeded in inducing defensive behavior by these nymphs. In this paper, we describe the life cycle of *T. kashifolia*, the defensive behavior of the first-instar nymphs and when they are produced in the life cycle. Because there was a large variation in the size of their forelegs, we address the issue whether soldier-caste differentiation occurs in this species.

2. Materials and Methods

2.1. Study Organism. The aphid *Thoracaphis kashifolia* (Uye) (the species epithet has been misspelled as "*kashifoliae*"



(a) (b) (c) (d)

FIGURE 1: (a) Colonies of *Thoracaphis kashifolia* on leaves of *Quercus glauca* (Tama, Tokyo; 14 October 2015); (b) a woolly colony of *T. kashifolia* (Tama, Tokyo; 14 October 2015); (c) two apterous adults and one nymph to be aptera (upper right) of *T. kashifolia* (Tama, Tokyo; 8 February 2016); (d) defensive nymphs of *T. kashifolia* clinging to an experimentally introduced lepidopteran larva (Tama, Tokyo; 1 October 2015).

(a) (b)

FIGURE 2: Apterous generation of *Thoracaphis kashifolia* (Ome, Tokyo; 6 November 2013): (a) adult; (b) first-instar nymph. Scale bars: 100 μm.

[6, 24, 25] or "*kashiwae*" [26–28]) forms colonies on the upper surfaces of leaves of *Quercus glauca* (Figures 1(a) and 1(b)) in the south-western half of Japan [27, 28] and in Taiwan [24]. A record from *Q. acuta* [29] remains to be confirmed. The apterous adults are aleyrodiform (Figures 1(c) and 2(a)), sessile, and flattened and are found throughout the year on the leaves [27]. The colonies produce alates in autumn and become remarkably woolly like candy floss (Figure 1(b)) during this period. The life cycle is supposed to be anholocyclic, without returning to *Distylium racemosum* [6, 27], which is the only known primary host of Nipponaphidini in Japan [30–32]. In this paper, colonies that are covered with long wax filaments

Table 1: Collection data, the number of nymphs to be alates, the percentage of first-instar nymphs and their molting rate, and the most advanced stage of the alate generation for 23 woolly colonies.

Colony code	Collection place	Date of collection[†]	Number of nymphs to be alates[‡]	% first-instar nymphs to be alates[§]	Most advanced stage of the alate generation
15226	Tama, Tokyo	4 Sep. 2015	616 (722)	90.2[¶] (27.5)[¶]	2nd instar
15228	Tama, Tokyo	12 Sep. 2015	174 (265)	99.3[¶] (16.8)[¶]	2nd instar
15230	Tama, Tokyo	20 Sep. 2015	252 (315)	61.1 (9.1)	3rd instar
Exp#1	Tama, Tokyo	28 Sep. 2015 (29 Sep)	425 (532)	32.9 (1.4)	4th instar
Exp#2	Tama, Tokyo	28 Sep. 2015 (29 Sep)	922 (1150)	40.6 (2.3)[¶]	4th instar
Exp#3	Tama, Tokyo	28 Sep. 2015 (29 Sep)	460 (532)	27.4 (0.8)	4th instar
Exp#4	Tama, Tokyo	30 Sep. 2015 (1 Oct)	427 (549)	35.4 (1.3)	Adult
Exp#5	Tama, Tokyo	30 Sep. 2015 (1 Oct)	1203 (1483)	37.8 (1.8)[¶]	Adult
Exp#6	Tama, Tokyo	30 Sep. 2015 (1 Oct)	442 (568)	42.8 (4.8)	4th instar
Exp#7	Tama, Tokyo	30 Sep. 2015 (1 Oct)	548 (651)	40.9 (2.2)	4th instar
15241	Kyoto, Kyoto Pref.	5 Oct. 2015	160 (257)	31.3 (2.0)	Adult
14158	Tama, Tokyo	8 Oct. 2014 (10 Oct)	218 (377)	11.0 (0)	Adult
14160	Tama, Tokyo	8 Oct. 2014 (10 Oct)	240 (618)	56.7 (0)	Adult
15257	Tama, Tokyo	13 Oct. 2015	210 (255)	21.9 (4.3)	4th instar[††]
15258	Tama, Tokyo	13 Oct. 2015	258 (371)	24.0 (0)	Adult
15260	Tama, Tokyo	13 Oct. 2015	172 (267)	25.0 (2.3)	Adult
UE1013	Tsuchiura, Ibaraki Pref.	13 Oct. 2015	88 (180)	1.1 (0)	Adult
14167	Hachioji, Tokyo	26 Oct. 2014	81 (254)	6.2 (0)	Adult
14168	Hachioji, Tokyo	26 Oct. 2014	25 (92)	0	Adult
15271	Tama, Tokyo	27 Oct. 2015	85 (165)	1.2 (0)	Adult
15272	Tama, Tokyo	27 Oct. 2015	43 (112)	0	Adult
15273	Tama, Tokyo	27 Oct. 2015	74 (198)	1.4 (0)	Adult
15274	Tama, Tokyo	27 Oct. 2015	76 (157)	2.6 (0)	Adult

[†]Date of fixation in parenthesis. [‡]Colony size in parenthesis. [§]Percentage to all nymphs to be alates, with the molting rate (%) of first-instar nymphs in parentheses. [¶]Estimated from a subsample. [††]Alate adults had probably been produced in this colony.

(Figure 1(b)) are called "woolly colonies," while those that are not are "ordinary colonies" (colonies in Figure 1(a) but the central woolly colony).

2.2. Sampling of the Aphids. To investigate the annual life cycle and the colony composition, *T. kashifolia* was sampled from *Quercus glauca* in various months. We regarded the aphids on a single leaf as a colony and sampled colonies mainly in Tama, Hachioji and Ome, western Tokyo, Japan, in 2013–2016. Additional colonies were sampled in Tsuchiura (Ibaraki Prefecture), Kashihara (Nara Prefecture), and Kyoto (Kyoto Prefecture) in 2015. The whole colonies each were preserved in a 30 mL glass vial of 80% ethanol together with the leaf. Later, under a dissecting microscope in the laboratory, the aphids in 23 woolly colonies listed in Table 1 and 22 ordinary colonies in Table 2 were detached from the leaf, counted, and sorted into the following groups: (1) apterous adults, (2) first-instar nymphs to be apterae, (3) non-first-instar nymphs to be apterae, (4) alate adults, (5) first-instar nymphs to be alates, and (6) non-first-instar nymphs to be alates.

From some woolly colonies, emerged alates were collected and used for transfer experiments to confirm whether these alates are sexuparae or secondary migrants (Section 2.5). For the same purpose, some alates (22 alates collected in Tama on 30 September 2015, nine in Tama on 15 October 2014, and 12 in Ome on 6 November 2013) were confined, together with a piece of paper, in a 5 mL cotton-plugged glass vial to force their larviposition there. A few days later, after confirming first-instar nymphs walking in the vial, 80% ethanol was poured into it. These nymphs were slide-mounted (see Section 2.3), and it was determined whether they were of sexuals or virginoparae (i.e., whether their mothers were sexuparae or secondary migrants). To supplement this, ten alates from colony 15241 and ten alates from colony UE1013 (Table 1) were slide-mounted, and it was determined whether the embryos in their bodies were the same in morphology as the first-instar nymphs born in the glass vial.

2.3. Examination of Aphid Morphology. For slide preparation, aphids preserved in 80% ethanol were cleared in heated 10% KOH solution. These aphids were stained with either Evans' blue or acid fuchsine, dehydrated in a mixture of glacial acetic acid and methyl salicylate for one day, and mounted in balsam via a mixture of xylol–phenol and pure xylol.

Table 2: Collection data, the number of apterous adults and those of nymphs for 22 ordinary colonies.

Colony code	Collection place	Date of collection	Number of apterous adults	Number of nymphs to be apterae	Number of nymphs to be alates
15001	Tama, Tokyo	3 Jan, 2015	7	2	0
15003	Tama, Tokyo	3 Jan, 2015	3	5	0
16004	Tama, Tokyo	8 Feb, 2016	52	6	0
16006	Tama, Tokyo	8 Feb, 2016	13	1	0
15022	Tama, Tokyo	3 Mar, 2015	18	2	0
15119	Tama, Tokyo	21 Apr, 2015	6	4‡	0
15144	Kashihara, Nara Pref.	19 May 2015	7	42	0
15208	Tama, Tokyo	17 Jun, 2015	30	31	0
15218	Tama, Tokyo	27 Jul, 2015	56	17	0
15221	Tama, Tokyo	19 Aug, 2015	64	1‡	0
15223	Tama, Tokyo	19 Aug, 2015	95	1‡	0
15236	Tama, Tokyo	30 Sep, 2015	0	6‡	0
15237	Tama, Tokyo	30 Sep, 2015	0	4‡	0
15238	Tama, Tokyo	30 Sep, 2015	0	25‡	0
15239	Tama, Tokyo	30 Sep, 2015	0	23‡	0
14170	Tama, Tokyo	26 Oct, 2014	1†	52	0
14171	Tama, Tokyo	26 Oct, 2014	0	19	0
13163	Ome, Tokyo	6 Nov, 2013	0	3‡	0
13164	Ome, Tokyo	6 Nov, 2013	0	6‡	0
15322	Tama, Tokyo	16 Dec, 2015	33	3	0
15323	Tama, Tokyo	16 Dec, 2015	108	4‡	0
15324	Tama, Tokyo	16 Dec, 2015	13	6	0

†A teneral aptera.‡First-instar nymph(s) only.

Many slide-mounted specimens were examined under a light microscope. Since there seemed to be large variation in the sizes of their fore- and mid-legs, all or about 120–180 subsampled first-instar nymphs to be alates from the 23 woolly colonies listed in Table 1 were slide-mounted, and one of their fore-femorotrochanters was measured. For the first-instar nymphs in one colony (Exp#6 in Table 1), the length and width of one fore-femorotrochanter (defined in Figure 4(a)) were measured. Measurements were made using a digital camera (FX630; Olympus, Tokyo, Japan) equipped with image analysis software (FlvFs; Flovel, Tachikawa, Japan). It was also recorded whether these first-instar nymphs had the next instar cuticle developing inside (i.e., whether they were in the pharate stage). The percentage of nymphs in the pharate stage roughly corresponds to the degree of their sterility; if the defensive nymphs were completely sterile and did never molt, no nymphs with the next instar cuticle would be found. All non-first-instar nymphs to be alates in two colonies (Exp#1 and 14160 in Table 1) were also slide-mounted to know the composition of instars and whether they were in the pharate stage.

2.4. Defensive Test. To confirm whether aphid nymphs with enlarged legs would really attack other insects, the following experiment was carried out. A total of seven leaves with woolly colonies, which seemed to have produced defensive nymphs, were carefully removed from trees of *Quercus glauca* in Tama on 28 and 30 September 2015. They were each kept in a plastic container with a sheet of paper, and on the day of collection, two lepidopteran larvae (collected from leaves of *Broussonetia kazinoki*; ca. 3–8 mm) were placed on each colony. When an introduced larva was attacked by aphids within a short period, the larva was placed on a sheet of paper and the attacking behavior was recorded by a video camera attached to a dissecting microscope. After taking a video, the larva and aphid nymphs clinging to it were deposited in a vial of 80% ethanol. When introduced larvae were not attacked by aphids, they were left in the container under a room temperature for approximately 24 hours and examined and deposited in 80% ethanol together with aphids clinging to them. Nine out of 11 larvae attacked by defensive nymphs were macerated in 10% KOH solution, stained with acid fuchsine, and mounted on a glass slide together with the nymphs to examine whether the nymphs really pierced the larval skins under a light microscope. Aphid nymphs that attacked the remaining two larvae were slide-mounted after being detached from the larvae. The remaining aphids were preserved in vials of 80% ethanol, and later defensive first-instar nymphs and other morphs were sorted and counted under a dissecting microscope as mentioned in Section 2.2.

2.5. Transfer Experiment. To confirm whether alates of *Thoracaphis kashifolia* are secondary migrants (alate virginoparae), a transfer experiment was carried out with a test

tree (ca. 2.5 m tall, 5 cm in diameter at 50 cm height) of *Quercus glauca* planted in a garden in Tama. The tree was free from *T. kashifolia*. Eight colonies of *T. kashifolia* were collected from other trees of *Q. glauca* in Tama and Hachioji on 13 October 2015 and kept in plastic containers. Many alates emerged from these colonies. Five twigs of the test tree were chosen and covered with a nylon bag (ca. 50 × 90 cm), and a total of 570 alates were put into the five bags on 14 and 18 October. All leaves in the five bags were examined on 24 October 2015, 22 November 2015, and 7 February 2016.

2.6. Data Analysis. The comparison of fore-femorotrochanteric length between attacking and non-attacking first-instar nymphs was performed by *t*-test, after checking the data for normal distribution (Kolmogorov–Smirnov test) and homogeneity of variance (*F*-test). Differences in the molting rate between the first instar and the remaining three instars were analyzed using Fisher's exact test with Holm's correction for multiple comparisons. Differences in the molting rate between first-instar nymphs with longer forelegs and those with shorter forelegs within three colonies (15226, 15228, and 15230) were analyzed by median test with Fisher's exact test. For the relationships between the fixation date of colonies (collected in Tama in 2015) and the proportion of molting first-instar nymphs, we used a generalized linear model with binomial errors. All statistical analyses were performed with the software R v3.2.3 [33].

3. Results

3.1. Occurrence of Defensive Nymphs. Colony size and composition of our samples are listed in Tables 1 and 2. Ordinary colonies, or colonies that were not covered with long wax filaments, contained only one kind of first-instar nymphs which were small and had ordinary fore- and mid-legs and short marginal setae on the abdomen (Figure 2(b)). Such colonies were seen throughout the year (Table 2), and there is no doubt that these first-instar nymphs would grow to sessile apterae (Figures 1(c) and 2(a)). Colonies covered with long wax filaments, or woolly colonies (Figures 1(a) and 1(b)), were seen from September to early November. In these colonies (except those collected late in the season) there were first-instar nymphs of another kind: they were larger than first-instar nymphs to be apterae and had enlarged fore- and mid-legs with large, strongly curved claws, and long marginal setae on the abdomen (Figure 3(a)). Our analysis of colony composition revealed that these first-instar nymphs would grow to alates through wing-padded nymphal stages and that the first- to fourth-instar nymphs to be alates excreted long wax filaments that made the entire colony like candy floss (Figure 1(b)). Defensive nymphs were first-instar nymphs to be alates (see below).

3.2. Defensive Behavior. In all seven colonies onto which two lepidopteran larvae were introduced, one or both larvae were attacked by defensive nymphs (Figure 1(d), Table 3). Three of the 14 larvae were found hidden under the paper sheet and seemed to have escaped from being attacked by aphid nymphs, while the remaining eleven were attacked by one to

(a) (b)

FIGURE 3: First-instar nymphs of the alate generation of *Thoracaphis kashifolia*: (a) typical defensive nymph with enlarged fore- and mid-legs (Tama, Tokyo; 29 September 2015); (b) first-instar nymph produced early in autumn (Tama, Tokyo; 4 September 2015). Scale bars: 100 μm.

60 nymphs (115 in total). Four nymphs were detached from the larva after being deposited in ethanol, but the remaining 111 were still tightly clinging to the larva. All 115 nymphs were first-instar nymphs to be alates and in the nonpharate stage. It was observed under a dissecting microscope that defensive nymphs were tightly clinging to the lepidopteran larvae with their enlarged mid- and forelegs and seemed to pierce them with their stylets. When attacked, the larvae twisted their bodies and bit off parts of some nymphs clinging to the larvae with their mouthparts (supplementary video 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/4036571). Examination of the slide-mounted specimens of nine lepidopteran larvae attacked by aphid nymphs revealed that the stylets of 11 attacking nymphs were inserted in the bodies of the larvae (Figure 5). The maximum length of the stylets inserted in the larvae was 170 μm; it was approximately as long as a fore-femorotrochanter of a small defensive nymph. The seven colonies also contained 17–76 (mean 39.3) first-instar nymphs to be apterae, but none of them joined to attack the lepidopteran larvae.

3.3. Size and Molting Rate of Defensive Nymphs. There was a large variation in the size of fore- and mid-legs of first-instar nymphs to be alates. Some had distinctly enlarged fore- and mid-legs (Figures 3(a), 4(a), and 4(b)) like *Colophina* soldiers, while some had only slightly enlarged or nearly ordinary legs (Figures 3(b) and 4(c)) and others were intermediate between the two. The length and width of one fore-femorotrochanter are shown as a scattered diagram for almost all first-instar nymphs in a colony (Exp#6) used in the defensive test: 31 nymphs to be apterae and 186 nymphs to be alates including 62 that actually attacked the introduced moth larvae (Figure 6). The 62 nymphs that attacked the larvae had forelegs of various sizes (Figure 6) and their fore-femorotrochanters

Table 3: Results of introducing lepidopteran larvae onto seven colonies.

Colony code	Lepidopteran larva: code	Lepidopteran larva: length in mm	Date and time of introduction	Date and time of confirmation	Number of aphids that attacked the larva*
Exp#1	1a	5	28 Sep., 16:00	29 Sep., 15:00	2
Exp#1	1b	4	28 Sep., 16:00	29 Sep., 15:00	0
Exp#2	2a	5	28 Sep., 16:00	28 Sep., 16:05	1
Exp#2	2b	5	28 Sep., 16:00	29 Sep., 16:00	9
Exp#3	3a	4	28 Sep., 16:00	28 Sep., 17:30	8
Exp#3	3b	6	28 Sep., 16:00	29 Sep., 15:00	5
Exp#4	4a	4	30 Sep., 15:50	1 Oct., 15:00	2
Exp#4	4b	3	30 Sep., 15:50	1 Oct., 15:00	5 (1)
Exp#5	5a	5	30 Sep., 15:50	1 Oct., 15:00	0
Exp#5	5b	6	30 Sep., 15:50	1 Oct., 15:00	19
Exp#6	6a	7	30 Sep., 15:50	30 Sep., 16:50	60 (3)
Exp#6	6b	?	30 Sep., 15:50	1 Oct., 15:00	2
Exp#7	7a	3	30 Sep., 15:50	1 Oct., 18:00	2
Exp#7	7b	8	30 Sep., 15:50	1 Oct., 18:00	0

*Number of aphids that were detached from the larva after being deposited in alcohol is in parenthesis.

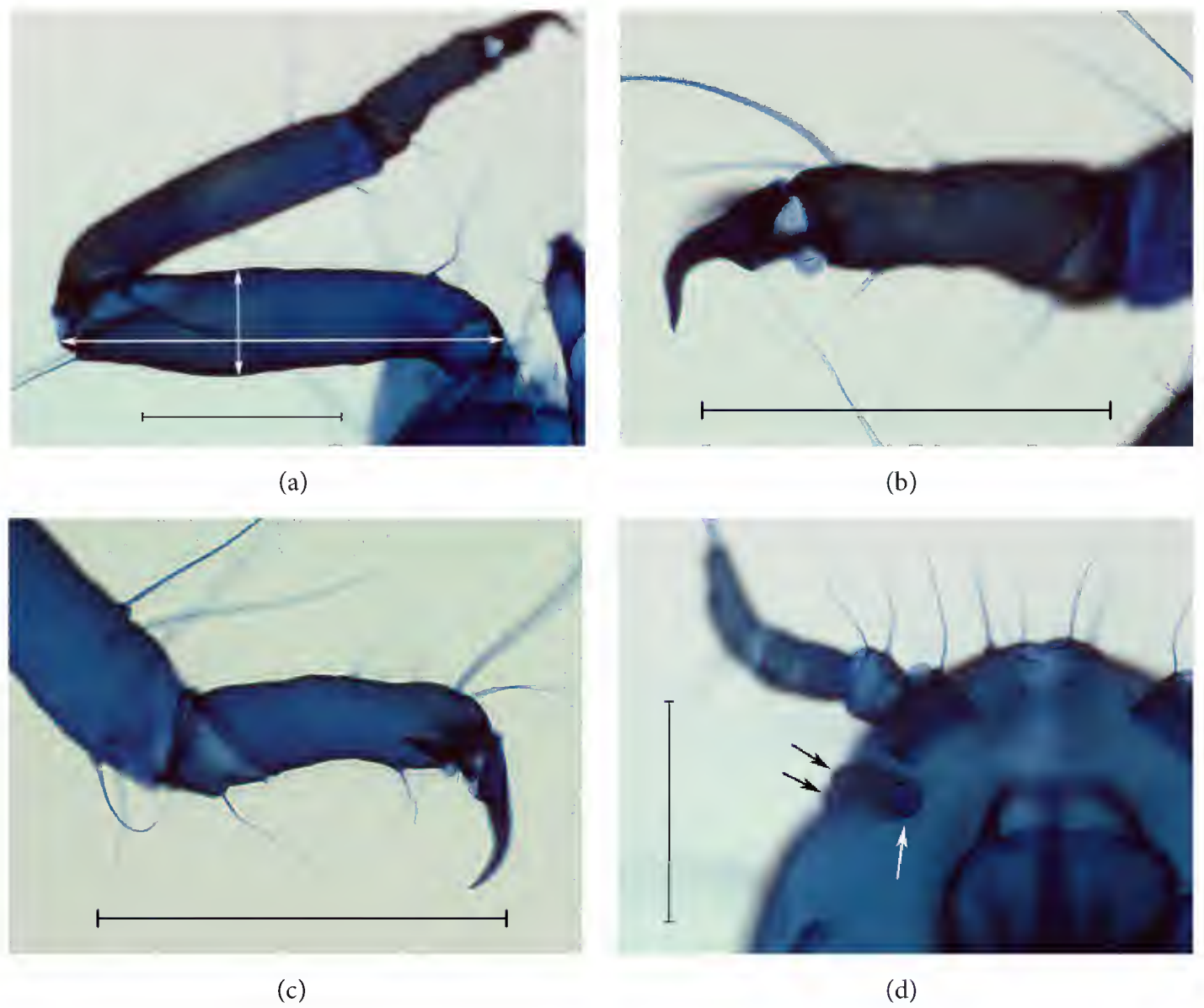


Figure 4: Forelegs and head of first-instar nymphs of the alate generation: (a) an entire foreleg of a typical defensive nymph, with double-headed white arrows indicating the length and width of a femorotrochanter defined in this paper; (b) fore tarsus of a typical defensive nymph with thick, strongly curved claws; (c) fore tarsus of a nymph produced early in autumn with slender claws; (d) head of a typical defensive nymph (ventral focus) indicating the downward-directed facet (by a white arrow) and the positions of the remaining two facets (by black arrows) of the left triommatidium. Scale bars: 100 μm.

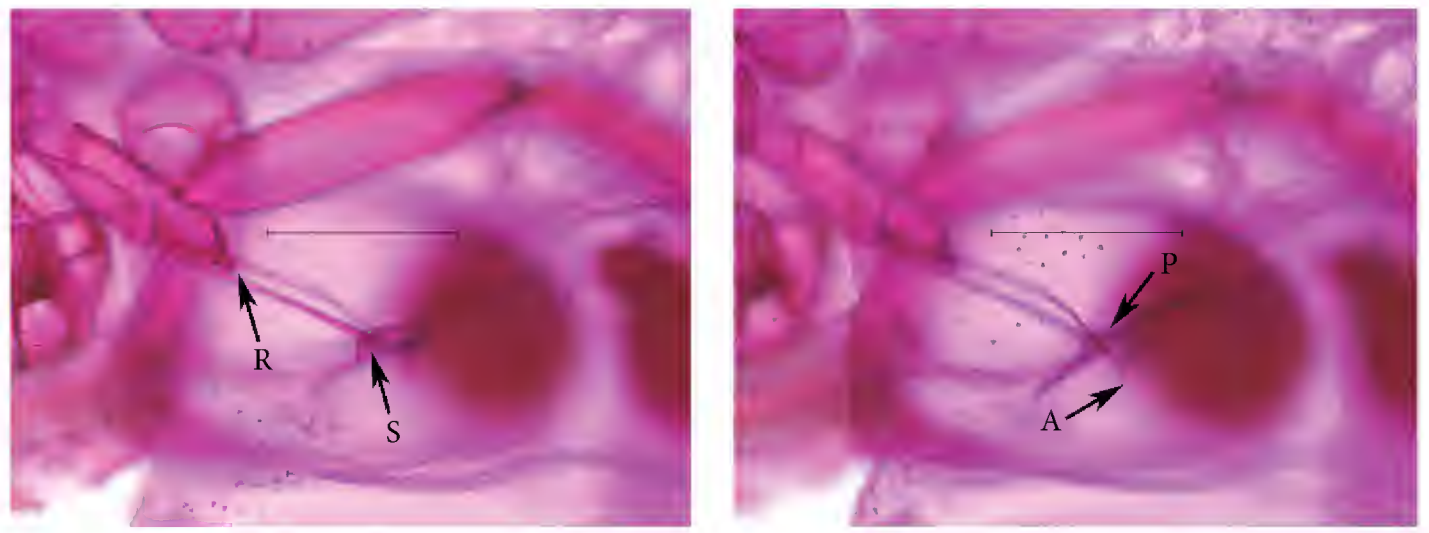


FIGURE 5: Stylets of a defensive nymph stuck through the epidermis of a lepidopteran larva: (a) upper focus; (b) lower focus. Capital letters on the figure indicate the apex of the rostrum of the attacking aphid (R), the socket of a seta on the epidermis of the lepidopteran larva (S), the apices of the aphid's stylets (A), and the presumed puncture point (P). The stylets are extended from the apex of the rostrum (R) to the point A through the point P below the socket (S). Scale bars: 100 μm.

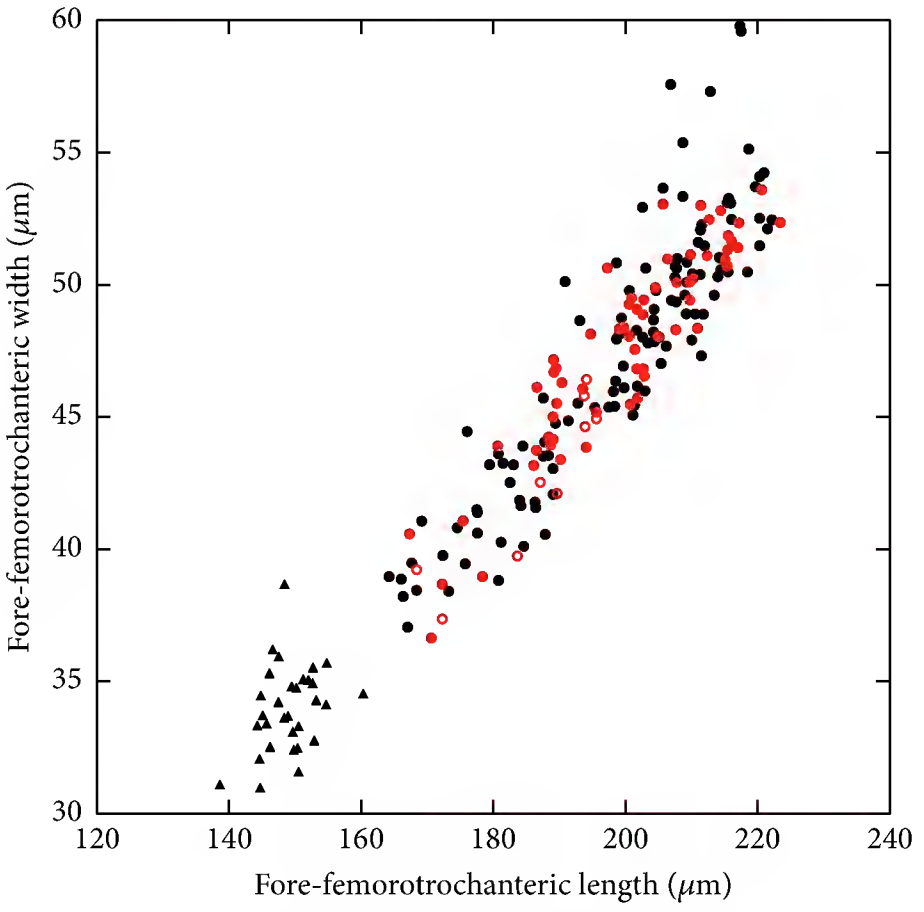


FIGURE 6: Scattered diagram of the length and width of one fore-femorotrochanter for 31 first-instar nymphs to be apterae (triangle) and 186 first-instar nymphs to be alates (circle) including 62 that actually attacked the introduced moth larvae (red closed circle) in a colony (Exp#6). First-instar nymphs (to be alates) that had the second-instar cuticle developing inside are indicated by red open circles.

were not longer than those of the remaining 124 nymphs that did not join the attack (t-test, $p = 0.418$).

First-instar nymphs to be alates in three colonies sampled early in the season (on 4, 12, and 20 September) had forelegs that were shorter than those sampled later in the season (Figure 7; see also Figure 8), and their "molting rates" (sensu Akimoto [11]), or the proportions of those nymphs that had the second-instar cuticle developing inside, were high, 27.5, 16.8, and 9.1%, respectively (Table 1). On the other hand, first-instar nymphs in the seven experimental colonies (colonies Exp#1–7) collected near the end of September and the colonies collected thereafter showed small values of the molting rate, less than 5%, and some (e.g., colonies 14158, 14160, and 15258) even zero percent (Table 1). Picking up 16 colonies collected in Tama in 2015 from Table 1, the molting rate of first-instar nymphs decreased with the date of fixation (generalized linear model with binomial errors, $\chi^2 = 117.5$, $p < 0.0001$). The molting rate of first-instar nymphs in colonies sampled near the end of September and thereafter, colonies Exp#1 and 14160, for example, was also lower when compared with the second- to the fourth-instar nymphs (Figure 9). (Note that in colony Exp#1, alate adults had not yet appeared. Because the colony was relatively young, the molting rate of the fourth-instar nymphs was low.)

In the early-sampled three colonies (for colony 15228, see Figure 8(a), left), first-instar nymphs with shorter forelegs tended to have the next instar cuticle developing inside (median test with Fisher's exact test; $p < 0.01$). In colonies sampled later in the season, the molting rate was so low that the tendency could not be confirmed statistically; however, defensive nymphs with large forelegs molt at least at times. In colony 15257 (sampled on 13 October 2015), two out of 46 first-instar nymphs to be alates had the next-instar cuticle; their fore-femorotrochanters were 217 and 216 μm long, the fifth and sixth longest in that colony.

3.4. Results of the Transfer Experiment. Offspring of the alates introduced into the five bags colonized on a few leaves in four bags. When examined on 22 November 2015, a total of 22 aphids (2 apterous adults and 20 nymphs to be apterae) were seen on the upper sides of seven leaves of *Quercus glauca*. On 7 February 2016, 15 apterous adults and one (presumably dead) nymph were found on four leaves. Although, taking the number of the mother alates (more than 500) into consideration, the success rate in colonization may be low, the result shows that the alates are not sexuparae but secondary migrants. All first-instar nymphs born in the glass vials were to be apterae, or of the same phenotype as in Figure 2(b). The ten alates (from colony 15241) collected in Kyoto and the ten

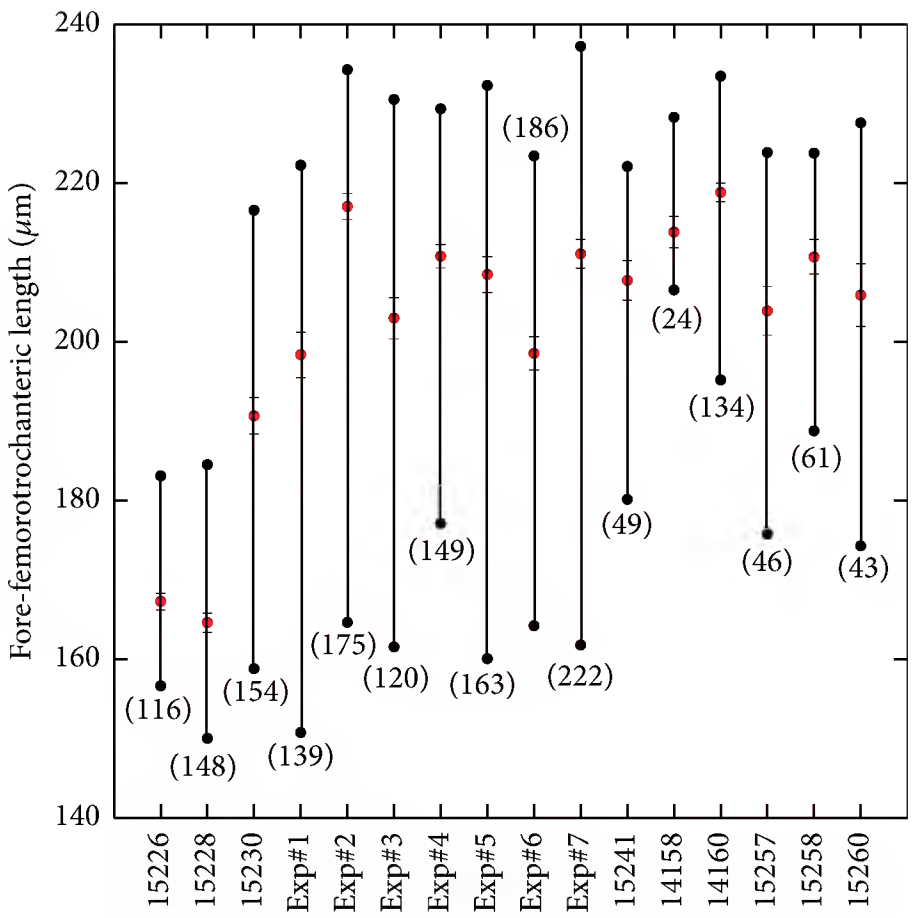


FIGURE 7: Lengths of fore-femorotrochanters of first-instar nymphs to be alates for 16 woolly colonies. Mean ± 2SE are shown by a red closed circle and two short horizontal bars on the vertical line which indicates the range. Number in parenthesis indicates the sample size. The colony codes shown below the horizontal axis are the same as in Table 1.

alates (from colony UE1013) collected in Tsuchiura also turned out to be secondary migrants. No sexupara was found in this study.

3.5. Predators Found in the Field. Colonies of *Thoracaphis kashifolia* were rather free of predators. From the 23 sampled woolly colonies and the 22 ordinary colonies we found no predator but a few dead syrphid eggs; it is not certain whether they were killed by defensive nymphs. Only one predator we found from woolly colonies was a hemerobiid larva (ca. 9 mm long). When found (in Tama on 14 October 2015), it was still alive but three defensive nymphs were tightly clinging to near the tip of its abdomen and one clinging to its thorax, near the base of its left mid-leg. We also found a chrysopid larva (ca. 10 mm long) from an ordinary colony, with a dead apterous adult of *T. kashifolia* on its back (in Tama on 19 August 2015). No ants were seen attending woolly or ordinary colonies.

4. Discussion

4.1. Convergent and Peculiar Features of the Defensive Nymph. As described in Section 3.2, first-instar defensive nymphs of *Thoracaphis kashifolia* clasp a predator with their enlarged fore- and mid-legs and pierce it with their stylets. Such attacking behavior is also known in soldiers of the eriosomatine *Colophina clematis* [1]. Their thickened legs with large, strongly curved claws (Figures 4(a) and 4(b)) are similar to those of the soldiers of *Colophina* spp. in shape (cf. [34, 35]), which is no doubt due to convergent evolution. On the other hand, the defensive nymphs of *T. kashifolia* have an idiosyncratic attacking device. The aphids of the tribe Nipponaphidini on the secondary host have long stylets, which are far longer than the rostrum [36], like adelgids [37]. When not extended, the stylets are kept coiled in the head; it is not easy to measure how long they are. In one slide-mounted defensive nymph, the stylets were extended about 330 μm from the apex of the rostrum. Defensive nymphs often inserted their stylets deep in the body of a lepidopteran larva to which they were clinging; the length of the stylets inserted in the larva was up to 170 μm (Section 3.2). Such deep insertion of stylets has been unknown from defensive nymphs of other groups. Defensive nymphs of *T. kashifolia* may use the stylets inserted in the body of an enemy as an anchor, so as not to be detached easily from it.

Another peculiar feature of the defensive nymph of *T. kashifolia* is its eyes. An aphid nymph of Hormaphidinae has a pair of triommatidia, each of which consists of three facets. In the first-instar nymphs of *T. kashifolia* (both nymphs to be apterae and to be alates), one facet is located apart from the remaining two, on the underside of the head and directed downward (Figure 4(d)). The same type of triommatidia is known in the first-instar nymphs of *Metathoracaphis isensis* [31], which also forms colonies on the upper sides of leaves of the host oak (*Quercus gilva*). This suggests that such triommatidia may be an adaptation in the life on the upper side of a leaf and that the downward-directed facets might enable the nymphs living on the upper side to perceive enemies on the underside through the leaf tissue.

4.2. Soldier-Caste Differentiation? Two morphologically distinct phenotypes of first-instar nymphs occur in *Thoracaphis kashifolia*: first-instar nymphs to be apterae (Figure 2(b)) and those to be alates (Figure 3). The former are clearly smaller than the latter as exemplified in Figure 6. Such dimorphism also occurs in the nipponaphidine *Neothoracaphis quercicola* [38], *N. yanonis* and *N. saramaoensis* (our unpublished results), and *Reticulaphis* sp. [39], all of which produce tiny, flattened apterae on leaves and may have evolved in association with the miniaturization and/or flattening of their aleyrodiform apterae. Coexistence of two morphologically distinct generations in a colony where first-instar nymphs of one generation play a defensive role is already known in the gall-forming aphid *Pemphigus spyrothecae* [40, 41]. The issue we address below is whether caste differentiation occurs within the first-instar nymphs of the alate generation, and not between the apterous and alate generations.

As mentioned in Section 3.3, first-instar nymphs produced early in September had smaller fore- and mid-legs than those produced later. Also, their molting rate was higher than the latter. These facts indicate that a kind of soldier-caste differentiation occurs in the alate generation of *T. kashifolia*; first-instar nymphs as shown in Figure 3(a) are soldiers and those in Figure 3(b) are to be reproductives. However, there were many first-instar nymphs with forelegs of intermediate sizes (Figure 7), and nymphs with forelegs of various sizes attacked lepidopteran larvae (Figure 6). In addition, although the molting rate of first-instar nymphs produced late in the season was extremely low (from less than 5% to zero), some still tried to molt to the next instar. The demarcation

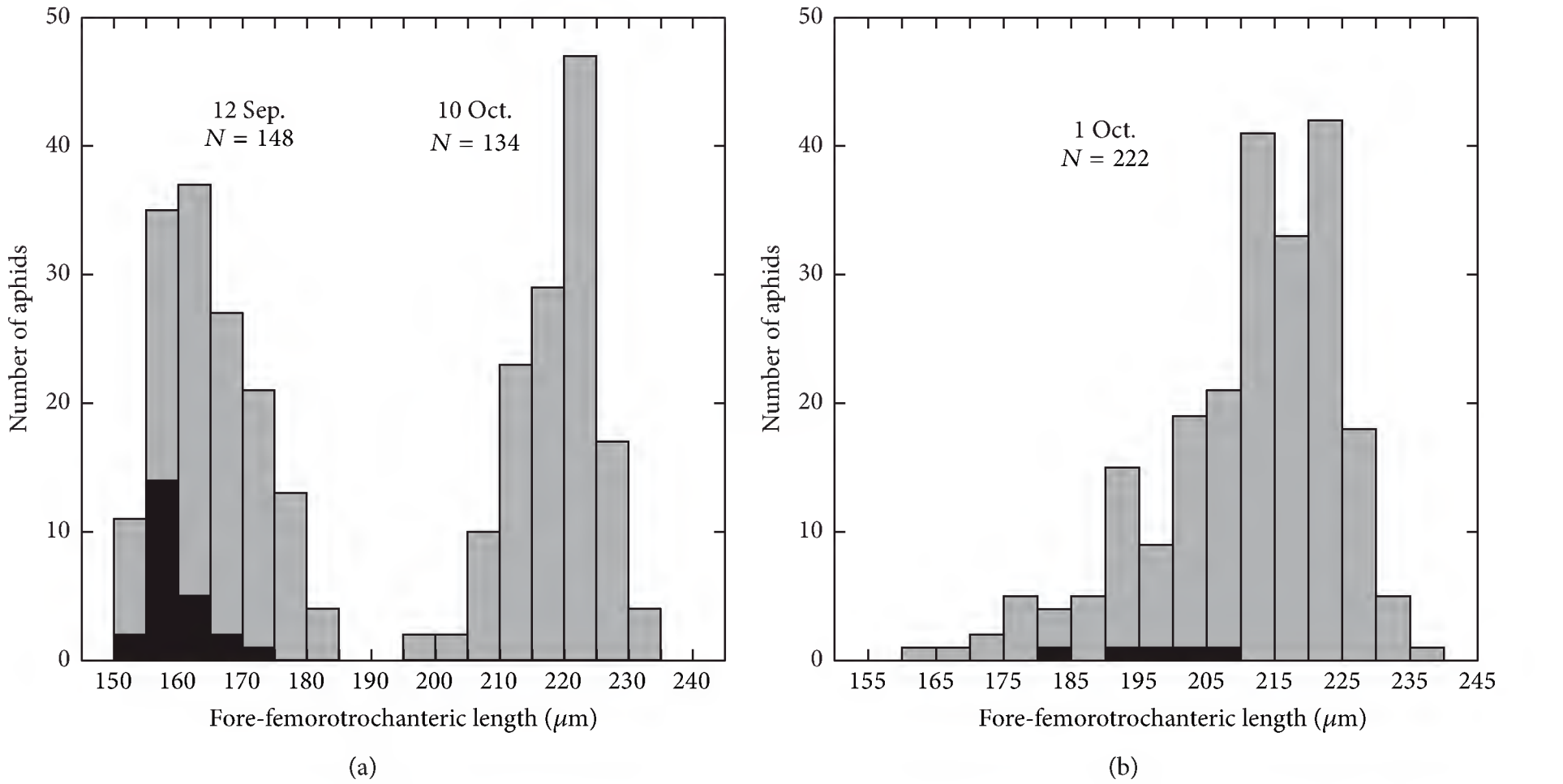


FIGURE 8: Frequency distribution of the fore-femorotrochanteric length of first-instar nymphs to be alates with (indicated by black) or without (indicated by grey) the next-instar cuticle developing inside. (a) Colony 15228 (left) on 12 September 2015 and colony 14160 (right) on 10 October 2014; (b) colony Exp#7 on 1 October 2015.

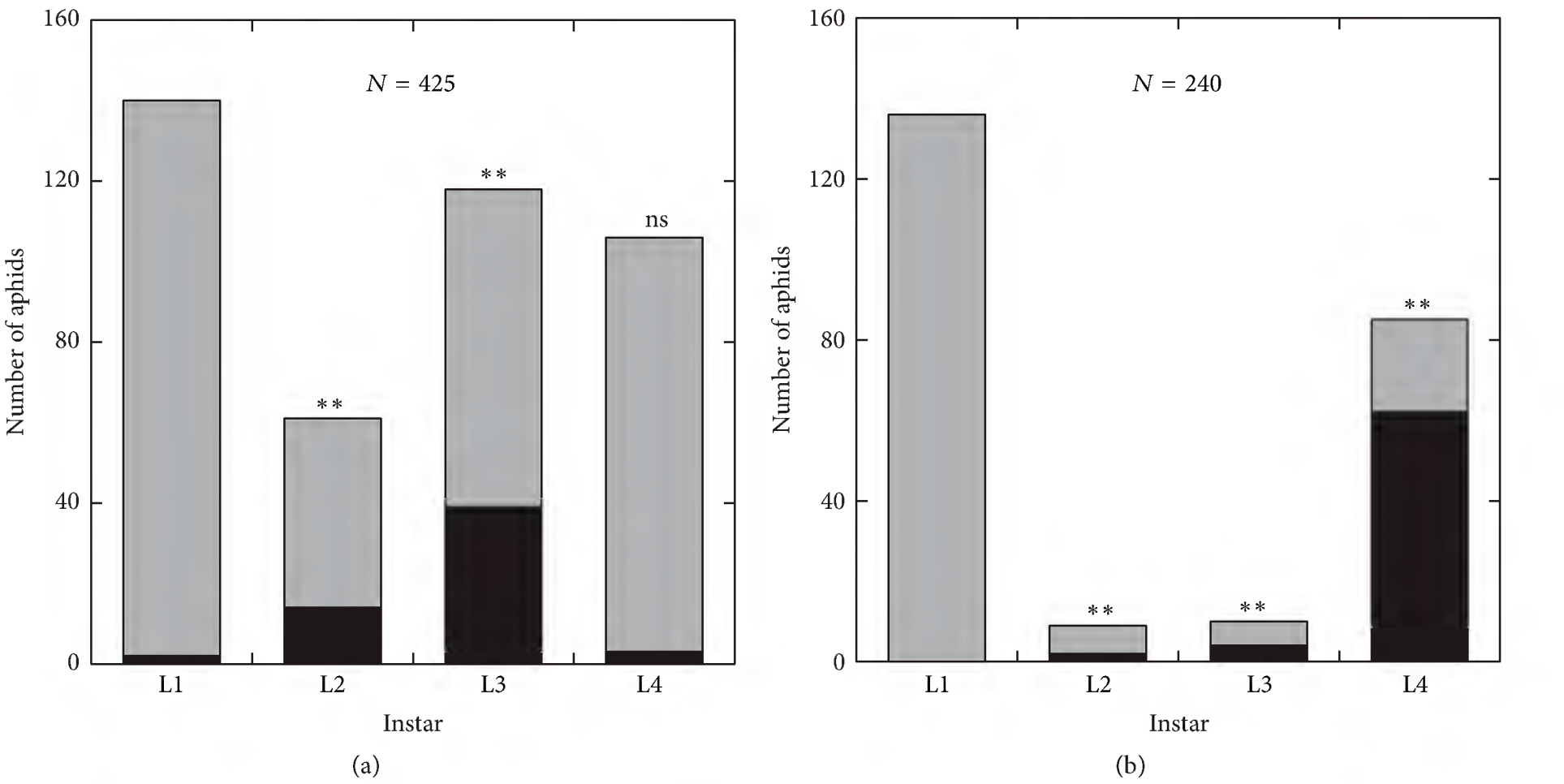


FIGURE 9: Number of the first- to the fourth-instar nymphs with (indicated by black) or without (indicated by grey) the next-instar cuticle developing inside. (a) Colony Exp#1 (29 September 2015); (b) colony 14160 (10 October 2014). L1, L2, L3, and L4 denote the first-, second-, third-, and fourth-instar nymphs, respectively. Significant differences ($p < 0.01$) in the molting rate between the first instar and the remaining three instars are indicated by double asterisks above each column (Fisher's exact test with Holm's correction).

between the two castes is therefore not clear cut. This is an incipient kind of soldier differentiation, or colonies of *T. kashifolia* are at an intermediate state between those with sterile soldiers (as in *Colophina clematis* [1]) and those with "monomorphic" defensive nymphs which all are supposed to have the potential to grow into reproductives (as in *Hemipodaphis persimilis* [11] or *Hamamelistes* spp. [13, 42]).

4.3. Life Cycle of Thoracaphis kashifolia in Japan. The present study confirmed that the life cycle of *Thoracaphis kashifolia*

is anholocyclic in Japan, as had been suggested by Takahashi [27]. That is, aleyrodiform apterous adults propagate themselves by parthenogenesis throughout the year on leaves of the oak *Quercus glauca*. They give birth to first-instar nymphs that grow to alates from early September. These alates are secondary migrants; they migrate to trees of *Quercus glauca*. First-instar nymphs of the alate generation play a defensive role. During October, the number of the alate generation decreases steeply. The seven colonies used in the defensive test (fixed on 29 September and 1 October) contained 425–1203 (mean 632) nymphs to be alates, while six colonies sampled on 26 and 27 October contained only 25–85 (mean 64) such nymphs (Table 1); the number of nymphs decreased to approximately 1/10 during 25–28 days. The number of first-instar nymphs to be alates decreased even more steeply, to 6/1000. This steep decrease could not be explained by molting of the first-instar nymphs, because the molting rate during this period was very low (Table 1, Figure 9). It was no doubt due to the high mortality of the defensive nymphs in exposed colonies.

We still do not know whether or how often a new colony of *T. kashifolia* begins with a single aphid. Our preliminary (unpublished) observations showed that some new colonies were formed on leaves near a woolly colony by more than one first-instar nymph to be aptera (e.g., colonies 15236–9 in Table 2). Although new colonies will also be formed by alates (Section 3.4), we have not yet confirmed this type of colony founding under natural conditions.

5. Conclusion

In this paper we made it clear that an incipient soldier-caste differentiation occurs in the alate generation of the aphid *Thoracaphis kashifolia*. This is the first discovery of defensive individuals from Nipponaphidini on their secondary hosts. The discovery was a little surprise because apterous adults of this group are sessile and seem to be protected by the hard exoskeleton. The defensive individuals are first-instar nymphs of the alate generation and therefore are produced only when aphids of the alate generation are present.

Competing Interests

The authors have no affiliations or involvement with any organization that has a financial interest in the results discussed in this paper.

Acknowledgments

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Research Article

Abrupt Geographical Transition between Aposematic Color Forms in the Spittlebug *Prosapia ignipectus* (Fitch) (Hemiptera: Cercopidae)

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Over most of its range populations of the spittlebug *Prosapia ignipectus* (Fitch) (Hemiptera: Cercopidae) are monomorphic for black dorsal coloration. At the far northeastern margin of the species range in Maine, a cluster of populations is monomorphic for the presence of traverse orange dorsal lines against a black background. The narrow gap separating monomorphic black and monomorphic lined populations is less than 10 km wide, shows no evidence of a hybrid zone, and is without consequential physical barriers or ecological breaks. This sharp and unexpected division of color forms seems to have persisted for at least 90 years. It appears to be the sharpest divide ever recorded between geographically adjacent populations monomorphic for alternative aposematic color forms. About 45 kilometers to the southwest of this dividing line, three closely situated populations, surrounded by monomorphic black populations, are polymorphic for the two color forms. These observations are at variance with several expectations for aposematic species: (1) that local populations will be monomorphic for warning coloration, (2) that adjacent populations monomorphic for different local color forms will be linked by populations with mixed or hybrid forms, and (3) that geographic boundaries between contrasting aposematic color forms should be temporally unstable.

1. Introduction

Many organisms have conspicuous coloration that warns potential predators of strong defenses, such as toxic chemical compounds, potent venom delivery systems, or particularly effective escape mechanisms [1, 2]. This aposematic, or warning, coloration signals to potential predators that a particular species is not a desirable subject for pursuit. Individual predators learn to avoid aposematic prey through initial unpleasant or unfruitful encounters, sparing subsequently encountered individuals with similar warning coloration from predation. This results in positive frequency-dependent selection for conspicuous coloration. As a result, local populations should become monomorphic for a single color form [1–3] and, by extension, closely adjacent populations should over time become monomorphic for the same color form through displacement of one form by another [4–6]. Here we detail the existence of local populations of the spittlebug *Prosapia ignipectus* (Fitch) that violate these expectations.

The genus *Prosapia* has 14 species distributed from the northeastern United States (and far Southern Ontario, Canada) to Colombia [7–9]. It falls within the family Cercopidae (Hemiptera: Cercopoidea), a large, predominantly tropical group with about 475 New World species [9, 10]. Only two species of Cercopidae are found in the USA (Figure 1): *Prosapia ignipectus* (Fitch) (Figures 2(a) and 2(b)), the northernmost representative of the family in the New World, and *Prosapia bicincta* (Say) (Figures 3(a) and

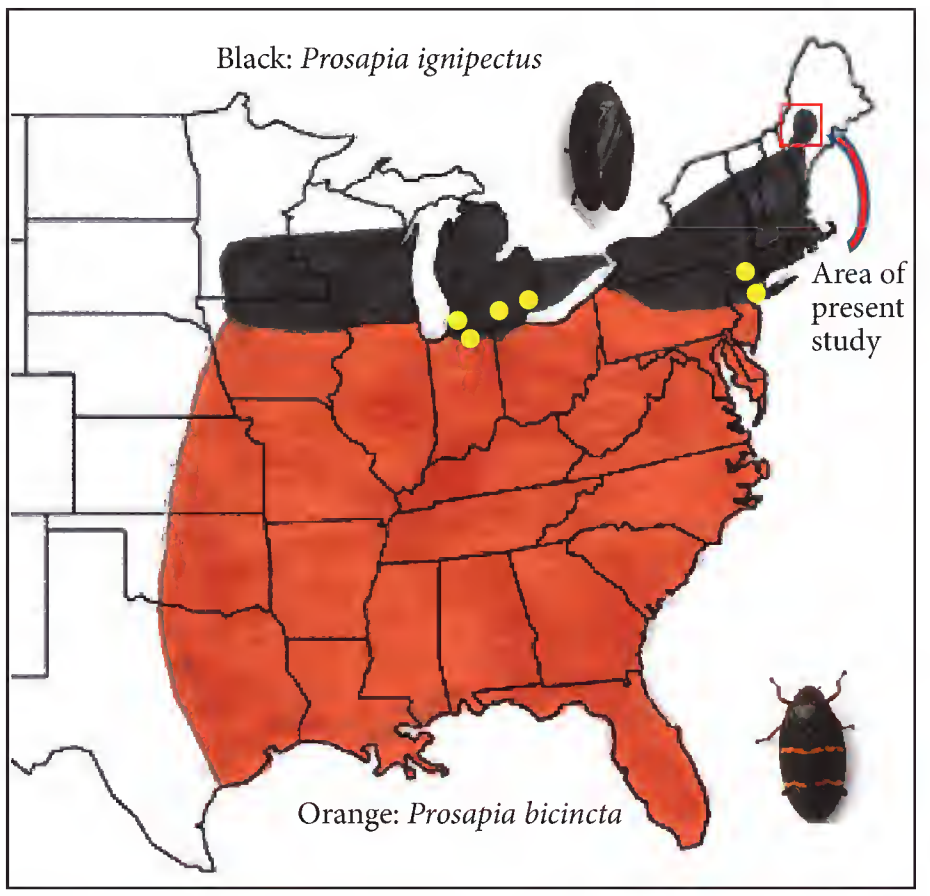


FIGURE 1: Approximate species ranges of *Prosapia ignipectus* (in black) and *Prosapia bicincta* (in orange). The populations examined in this study lie in the far northeastern portion of the *P. ignipectus* range in the area of the state of Maine outlined in red (see Figure 6). Yellow dots represent the occurrence of populations with mixed color forms, nominally all *P. ignipectus*, towards the southern part of the *P. ignipectus* range (see text for details).

3(b)), a pasture pest in the southeastern USA [11]. The two species are morphologically similar. *P. ignipectus* was originally described by Fitch [12] as *Monecphora ignipecta*. It was synonymized as a variety of *Monecphora bicincta* by Van Duzee [13]. Fennah designated *P. bicincta* as the type species of the newly created genus *Prosapia* [14] and later included the form *ignipecta* as a geographical subspecies of *P. bicincta* [15]. Hamilton reinstated *P. ignipectus* as a distinct species [7].

P. ignipectus is distributed across the northeastern USA from Minnesota to Maine and in the southernmost portion of Ontario, Canada. *P. bicincta* is distributed in the southeastern USA from Oklahoma to Florida (Figure 1). The two species are distinguished by geographical distribution, by subtle differences in the male genitalia [7], and by differences in host plant associations. *P. ignipectus* is monophagous on the late season C4 perennial grass *Schizachyrium scoparium* (Michx.) Nash, Little Bluestem [16, 17]. Nymphs live on Little Bluestem roots. Adults emerge in late July or early August and feed on stems of the host [18, 19]. Although the range of Little Bluestem broadly overlaps the range of *P. bicincta* (compare Figure 1 with [20]), *P. bicincta* has never been reported on Little Bluestem and appears to be absent from Little Bluestem in New Jersey (VT observations) and North Carolina (J. Urban, pers. comm.). *P. bicincta* is polyphagous on a wide variety of C4 grasses [11, 17]. Nymphs live on the roots of host grasses and adults on the upper parts of the same hosts. Adult males of *P. bicincta* feed on ornamental hollies (*Ilex* spp.) in addition to C4 grasses [21].

Adults of both species exhibit reflex bleeding ([22] and Figures 4(a) and 4(b)). When molested they release an odiferous yellow liquid from their tarsi. This behavior deters some predators and is probably the primary basis for the colorful patterns widespread among the Cercopidae [22] (see examples of striking coloration in [9, 10]). Consistent with this behavior, both *P. bicincta* and *P. ignipectus* have dorsal coloration that is conspicuous in natural settings. *P. bicincta* individuals have a black dorsal surface traversed by a single narrow lateral orange line across the widest part of the pronotum and a pair of narrow orange lines across the elytra (Figure 3(a)). With exceptions described below, *P. ignipectus* individuals have uniformly black dorsal coloration (Figure 2(a)). Both species stand out against the grass stems on which they characteristically sit prominently exposed (Figures 5(a) and 5(b)). The abdomen of *P. ignipectus*, which is visible while flying, is predominantly red or reddish orange (Figures 2(b) and 5(b)), as are the legs, although there is variation in the intensity of the red and in its combination with darker tones on the legs and on the red and black patterned ventral surface. In contrast, red markings on the abdomen and legs of *P. bicincta* are more subdued (Figure 3(b)).

In 1920, while collecting in preparation for a report on the Orthoptera of Maine, Albert Morse stumbled across a *Prosapia* population in Norridgewock, Maine, which consisted entirely of individuals with *P. bicincta*-like dorsal coloration [18, 23]. Morse interpreted these specimens as a disjunct New England occurrence of *P. bicincta*. With the exception of one piece of contemporary work [19], Morse's observation of lined *Prosapia* in Maine went unremarked and uninvestigated for several decades. Many, including the authors, assumed that the report of lined specimens in Maine, about 500 km northeast of the known range of *P. bicincta*, was in error. However, reexamination of Maine populations has vindicated Morse's original observation, though not his interpretation, and disclosed an unusual case of polymorphic aposematism rivaling, on a smaller scale, some of the paradigmatic cases observed in the tropics [24].

2. Materials and Methods

In September 2003, VT sought Morse's original locality and found a population of lined *Prosapia* living on Little Bluestem 6 km north of Norridgewock, Maine. A second lined population was found 3.5 km north of nearby Solon Center, Maine. In 2004 VT revisited these locations and collected lined *Prosapia* in a number of nearby localities, as well as a large sample of black specimens in close proximity in New Vineyard, Maine. Examination of three historical Norridgewock specimens collected by Morse and preserved in the Museum of Comparative Zoology (MCZ) of Harvard University confirmed that lined individuals collected in 2003 and 2004 were indistinguishable from those Morse collected in the same locale more than 80 years before.

In 2006 VT made systematic investigations of the areas to the south, east, and northeast of Norridgewock. These observations confirmed that the populations in the vicinity of Norridgewock represent the northeasternmost populations of *P. ignipectus*. In 2012 VT and GC made an intensive survey

(a) (b)

FIGURE 2: *Prosapia ignipectus*, black form: (a) dorsal view and (b) ventral view. Photographs by Tom Murray.

(a)

(b)

FIGURE 3: *Prosapia bicincta*: (a) dorsal view and (b) ventral view. Photographs by Brandon Woo.

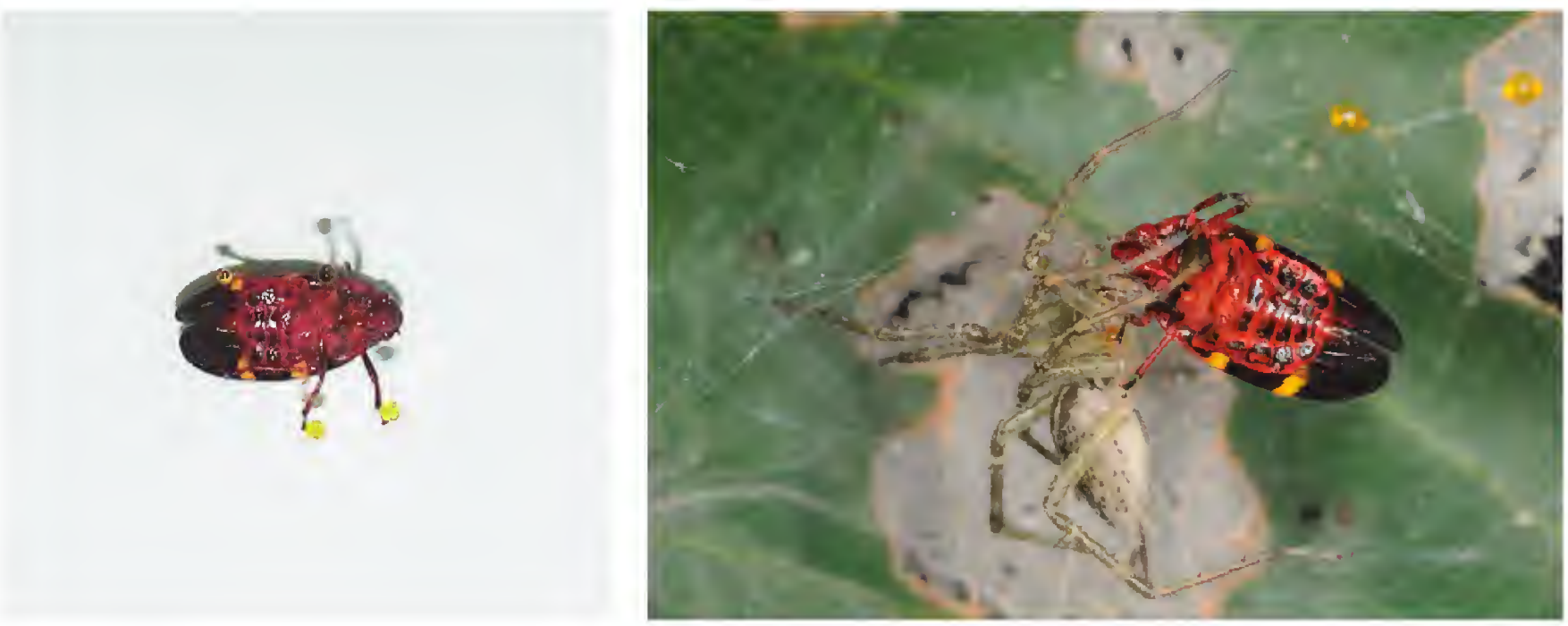
of Maine roadside populations of Little Bluestem from West Bethel, near the New Hampshire border, east to Norridgewock, making a special effort to determine the northern boundary of the *Prosapia* distribution and the location of the boundary between the lined and unlined populations. Little Bluestem is a particularly tractable substrate for collections. It grows in easily accessible clumps on the verges of public roads and is readily swept for collections. Specimens were preserved in 95% ethanol or mounted dry on pins. Voucher specimens have been deposited in the American Museum of Natural History (AMNH) and the MCZ.

In 2013 and 2014 VT swept Little Bluestem in New Jersey and New York in the vicinity of the eastern *P. bicincta*-*P. ignipectus* species boundary. In addition, GC and VT examined New York City area historical *Prosapia* specimens in the collection of the AMNH and specimens collected by James Bess in Indiana and Michigan near the local *P. bicincta*-*P. ignipectus* species boundary.

3. Results

3.1. Both the Lined and Black Maine Forms Are P. ignipectus, Which Is Distinct from P. bicincta. Morse believed that his Norridgewock specimens represented a geographically disjunct population of *P. bicincta*, a judgment apparently based solely on the presence of the lined orange pattern characteristic of *P. bicincta*. GC dissected genitalia of representative males of southern *P. bicincta* and black and lined specimens from the Maine populations in question. Male genitalia of the black and lined Maine forms are indistinguishable. Both differ from the genitalia of *P. bicincta* consistent with the subtle interspecific differences in the apices of the styles illustrated by Hamilton [7]. We conclude that both color forms belong to *P. ignipectus* and, in concurrence with Hamilton [7], that *P. ignipectus* is distinct from *P. bicincta*. The specific difference is also supported by subtle differences in shape between the species (*P. ignipectus* more slender and *P. bicincta* more robust), by the consistent difference in host plant associations noted above, and by the allopatric distribution of the taxa (Figure 1). DNA bar code analysis, based on sequences of a single mitochondrial gene, indicates that the species are closely related [25], probably reflecting recent speciation.

3.2. Populations Monomorphic for the Lined Form Are Uniquely Clustered at the Northeastern Species Margin and Separated from Populations Monomorphic for the Black Form by Less Than 10 km. Table 1 reports the color forms encountered in 32 *P. ignipectus* collections made in 2003, 2004, 2006, and 2012 between the New Hampshire border and Norridgewock. These populations represent the northeasternmost part of the species range. To the immediate south, between Norridgewock and the Atlantic coast, Little Bluestem appears to be absent. In some areas east and northeast of Norridgewock, for example, in parts of the Penobscot River Valley, Little



(a) (b)

FIGURE 4: Reflex bleeding in *Prosapia bicincta*: (a) droplets of yellow liquid emanating from tarsi, photograph by Sam Houston; (b) bleeding in response to attack by spider (see droplet of yellow liquid on front tarsus to left in picture and another droplet caught in the spider web in the background), photograph by Andy Williams.

(a) (b)

FIGURE 5: *Prosapia ignipectus*, lined form: (a) sitting characteristically exposed while feeding on stems of Little Bluestem and (b) mating among stems of Little Bluestem, female above and male below.

Bluestem is abundant but *P. ignipectus* is absent. In contrast, *P. ignipectus* is common on Little Bluestem from Norridgewock north almost to Bingham in the Kennebec River Valley and along some of its tributaries to the west as well as in a portion of the Androscoggin River Valley centering on the city of Rumford. North and west of these populations, occasional Little Bluestem patches without *P. ignipectus* extend up small river valleys into the Longfellow Mountains. The absence of *P. ignipectus* on Little Bluestem to the north and east indicates that factors other than host plant distribution limit its range.

Populations monomorphic for the lined form (Figures 5(a) and 5(b)) lie in a discrete cluster at the extreme northeastern edge of the species distribution (Figure 6). They encompass an area of about 675 km^2 bounded roughly by the towns of Norridgewock, Bingham, Carrabassett, and New Portland. Less than 10 km to the southwest in New Vineyard they are replaced by populations essentially monomorphic for the black form (with the exception of a single male individual taken in New Vineyard in 2004 that had a partially obscured single orange line across the pronotum) (Figure 6 and Table 1). These populations are linked to other monomorphic black populations to the southwest by a monomorphic black population in Wilton. The area in which the collections were taken varies in elevation from about 75 to 250 m and consists of low forest cover, broken by bogs, streams, human habitations, and farmland. There are no significant barriers to *P. ignipectus* dispersal and the roadside habitats in which *P. ignipectus* occurs are similar throughout the area. All of the monomorphic lined populations lie in the Kennebec River drainage, but this drainage also includes the monomorphic black populations in New Vineyard and Wilton. Monomorphic black populations to the immediate southwest lie in the Androscoggin River drainage (Figure 6).

3.3. A Geographically Limited Area Near Rumford Has Populations with Mixed Color Forms. Most of the populations along the Androscoggin River and its tributaries are monomorphic for the black color form, but three clustered populations,

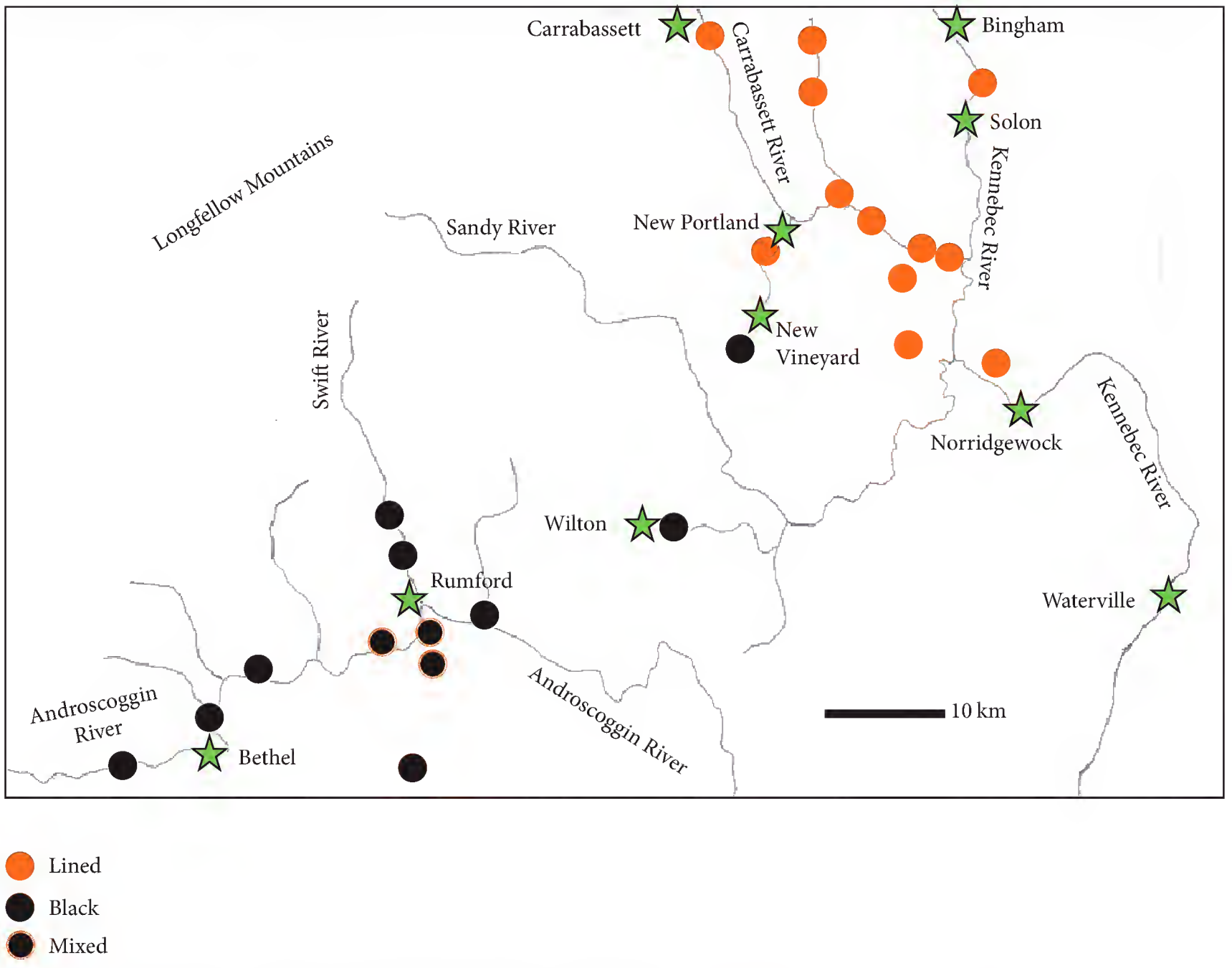


FIGURE 6: Distribution of *Prosapia ignipectus* populations with lined, black, and mixed dorsal color forms at the northeastern extremity of the species range in Maine. Some closely spaced localities sampled are represented by a single color symbol. In New Vineyard, a single partially lined specimen was observed among 114 specimens collected; the rest were black.

one in Rumford Center and two in nearby South Rumford on the opposite side of the river, exhibit a mixture of black, lined, and partially lined individuals (Figure 6 and Table 1). These populations are 45 kilometers from the nearest monomorphic lined population to the northeast. In each case the lined individuals are phenotypically variable: some fully lined and some partially lined, most of the partially lined specimens with a single line across the pronotum. All populations sampled in Maine to the south and southwest of Center Rumford as well as in adjacent parts of New Hampshire were monomorphic for the black color form (Figure 1 and VT unpublished observations), consistent with Morse's observations in Maine almost a century earlier [18].

3.4. A Few Populations near the P. bicincta-P. ignipectus Species Boundary Exhibit Mixed Color Forms. The AMNH collection includes one black specimen and one lined specimen (two elytral lines but no pronotal line) of *P. ignipectus* collected in Tuxedo Park, NY, in 1928. It also includes two lined specimens (one male and one female) collected along with two black specimens (one male and one female) on Staten Island, NY, in 1970. A sweep of Little Bluestem 20 km from Tuxedo Park by VT in August 2014 yielded 42 *P. ignipectus* specimens, all black. The Staten Island site appears to have been obliterated by residential development. Several other AMNH historical collections from northern New Jersey and Long Island, NY, consist entirely of specimens of the black color form.

In 1993–2000 James Bess collected short series of *P. ignipectus* specimens, some lined and some black, from Little Bluestem in fens near the Michigan-Indiana border, close to what appears to be the boundary between the *P. ignipectus* and *P. bicincta* distributions in the central Midwest (see [26] for background on the habitats and insect associations). At Liberty Fen in Jackson Co., MI, Bess collected a lined male and a black male. At Sawmill Fen in LaGrange Co., IN, he collected a lined male and a black female. At Indian Bowl Fen and nearby in Berrien Co., MI, he collected a lined male and a black male. At Brant Road Fen in Oakland Co., MI, he collected a lined male.

3.5. Additional Natural History Observations. Little Bluestem, a grass of nutrient-deficient soils, is an attractive host for spittlebugs, probably because it exhibits associative nitrogen-fixation [17]. During our sampling we searched for and found *Prosapia ignipectus* nymphs living slightly below ground level in Little Bluestem clumps, confirming

Table 1: *Prosapia ignipectus* collections in Maine, September 2003–August 2012. All specimens were swept from patches of Little Bluestem (*Schizachyrium scoparium* (Michx.)) growing on or near roadside verges.

Date	Abbreviated locality designation	County	Latitude N	Longitude W	Black ♂	Black ♀	Lined ♂	Lined ♀	Partially lined ♂	Partially lined ♀
10-ix-2003	Rte. 201A 6.5 km N. of Norridgewock	Somerset	44 44 54.72	69 50 07.67				11		
09-viii-2004	Rte. 201A 6.5 km N. of Norridgewock	Somerset	44 44 54.72	69 50 07.67			29	45		
09-viii-2004	Rte. 16 1 km SE of North New Portland	Somerset	44 54 57.37	70 00 30.33			9	42		
09-viii-2004	Rte. 27 1 km SE of New Portland	Somerset	44 52 32.54	70 06 48.90			2	3		
10-ix-2003	US 202 between Solon & Bingham	Somerset	44 59 00.16	69 52 01.34				5		
09-viii-2004	US 202 between Solon & Bingham	Somerset	44 59 00.16	69 52 01.34			31	15		
16-viii-2006	US 202 between Solon & Bingham	Somerset	44 59 00.16	69 52 01.34			2	10		
28-vii-2012	US 2 & Old Jay Street, Wilton	Franklin	44 35 00.34	70 14 07.21	8	10				
09-viii-2004	Rte. 27 & Lowell Drive, New Vineyard	Franklin	44 45 05.44	70 08 05.42	45	62			1	
28-vii-2012	Rte. 27 & Lowell Drive, New Vineyard	Franklin	44 45 05.44	70 08 05.42	3	1				
28-vii-2012	Rte. 27 & Basin Road S. of New Vineyard	Franklin	44 46 23.59	70 07 42.33	1	1				
28-vii-2012	Rte. 16 1 km W. of North Anson	Somerset	44 51 42.31	69 55 43.65			2	4		
09-viii-2004	Rte. 16 2 km W. of North Anson	Somerset	44 52 01.30	69 56 24.47			1	5		
28-vii-2012	Rte. 16 & Wentworth Road	Somerset	44 52 51.67	69 58 10.10			4	17		
28-vii-2012	Long Fall Dam Road 2 km N. of Sandy Stream	Somerset	44 59 25.20	70 03 05.35			19	17		
28-vii-2012	Long Fall Dam Road & Old County Road	Somerset	45 02 05.12	70 03 55.19			4			
28-vii-2012	Rte. 16 & 27 & Carriage Road, Carrabassett	Franklin	45 04 31.88	70 12 41.95			15	19		
30-vii-2012	US 2 W. of Bethel	Oxford	44 24 03.17	70 51 59.98	7	2				
30-vii-2012	Rte. 232 between Woodstock & Hanover	Oxford	44 26 11.71	70 39 16.17	1	2				
30-vii-2012	US 2 between Bethel & Newry	Oxford	44 27 57.07	70 48 02.33	5	3				
30-vii-2012	US 2 between Newry & Hanover	Oxford	44 29 04.92	70 43 54.22	1	1				
30-vii-2012	US 2 & Dragoons Road, Rumford	Oxford	44 31 00.39	70.33 36.73	4	4	3	2	2	2
30-vii-2012	US 2 near Dixfield	Oxford	44 31 52.17	70 29 03.67	1	2				
30-vii-2012	South Rumford Road, South Rumford	Oxford	44 32 03.43	70 36 26.13	1	1	1	1		1
30-vii-2012	Rte. 17 N. of Mexico	Oxford	44 35 27.50	70 33 41.72	6	1				
30-vii-2012	US 2 W. of East Wilton	Franklin	44 36 21.79	70 12 09.12	2	2				
30-vii-2012	Rte. 148 2 km W. of Rte. 43, Industry	Somerset	44 46 09.82	69 58 04.42			10	2		
30-vii-2012	Rte. 234 near Town Farm Road	Somerset	44 51 26.96	69 55 59.06			4	8		
13-viii-2012	Milton Rd. & Waite Road	Oxford	44 27 16.92	70 35 56.56	1	4				
13-viii-2012	Near intersect Skyway & Sunday River Roads	Oxford	44 28 16.10	70 50 26.78	9	6				
13-viii-2012	South Rumford Road near No View Farm	Oxford	44 28 56.45	70 38 16.23	4	1				
13-viii-2012	Wyman Hill Road, South Rumford	Oxford	44 31 09.95	70 31 40.24	4	16	1	1	2	
Total					*103*	*119*	*137*	*207*	*5*	*3*

♀: female; ♂: male.

speculation by Morse [18] and Boring [19] and a statement by Hamilton [16] that the nymphs are root feeders.

Also, while surveying the Little Bluestem associated populations recorded in Table 1, we collected four other spittlebug species, all of the family Aphrophoridae: *Philaenus spumarius* and *Neophilaenus lineatus* (both introduced from Eurasia) and the native spittlebugs *Lepyronia quadrangularis* and *Philaenarcys killa*. Only *L. quadrangularis* occurred in sizable numbers at multiple sites. It is clearly associated with Little Bluestem [17] but has a much broader host range [16]. *P. killa,* in contrast, is a Little Bluestem specialist [17]. In this study it cooccurred with *P. ignipectus* only in West Bethel, the westernmost Maine site sampled. It cooccurs frequently with *P. ignipectus* on Little Bluestem in Central New Hampshire (VT observations). *P. spumarius* occurs primarily on broadleaved herbs [16] and was likely associated with herbaceous dicots mingled with Little Bluestem. *N. lineatus* lives primarily on cool season C3 grasses [17] and was likely associated with C3 grass species mingled with Little Bluestem.

4. Discussion

The observation that *P. ignipectus* has populations monomorphic for alternative aposematic color forms sharply separated by only a few kilometers seems to be unique among polymorphic aposematic organisms. The closest parallels that have come to our attention form part of a complex Müllerian mimicry system involving the millipede genera *Apheloria* and *Brachoria* in the Appalachian Mountains of the USA. Marek and Bond [27] present detailed data suggesting that, in three of the seven species they investigated, populations monomorphic or nearly monomorphic for contrasting light and dark color forms occur in close geographical proximity. In the taxon *Apheloria* clade B a series of apparently monomorphic "chess" color form populations is separated from an apparently monomorphic "striped" color form population by only about 20 km. Similarly, in *Brachoria mendota* a series of populations that appear to be monomorphic for the "taillight" color form is separated by only 8–10 kilometers from populations that appear to be monomorphic for the contrasting "caution/road" color forms. Neither of these cases involves separation by a major mountain ridge, but a high ridge does separate a single population of *Brachoria dentata* monomorphic for the "headlight" color form from populations apparently monomorphic for the contrasting "caution/road" color form about 10 km distant. In many of these localities the sample sizes are small, but in each case the distributions suggest a sharp boundary of less than 10–20 km separating populations monomorphic or nearly monomorphic for contrasting aposematic forms.

In other reported cases, populations monomorphic for alternative warning color forms are clearly linked by mixed populations in the intervening territory. For example, in the bumblebee *Bombus melanopygus*, California populations monomorphic for red abdominal color are separated from Washington State populations monomorphic for black abdominal color by a 600 km cline of populations polymorphic for both [28, 29]. In Peru, populations of the poison-dart frog *Ranitomeya imitator* lying just 15 km apart are monomorphic for alternative aposematic color forms but are linked by intermediate mixed populations [30, 31]. In the well-studied Müllerian mimicry system involving the Neotropical butterflies *Heliconius erato* and *Heliconius melpomene*, populations of each species monomorphic for parallel mimetic forms are linked by hybrid clines [4, 32]. At one location in Peru, populations monomorphic for the "postman" and "ray" forms of the these butterflies are separated by a zone as narrow as 10 km [3], but in this and similar cases hybrid linking populations are the rule (see Figure 1 of [33]).

Sharp contact zones imply strong natural selection; the narrower the zone the stronger the selection [3]. The continued separation for at least 90 years of Maine lined and black populations in close proximity suggests that strong natural selection is maintaining homogeneity for the alternative color forms. Though there are no direct data bearing on their dispersal ability, *P. ignipectus* adults are strong fliers, making substantial gene flow over the short distance between monomorphic black and monomorphic lined populations not only possible but likely. In addition, there are indications that contact zones between aposematic forms may be temporally unstable and subject to a sweep of one form by another [4–6]. This raises two questions concerning the Maine populations: what special selective forces, if any, maintain the lined color form and why is the division between the forms so sharp and, apparently, stable? We say apparently stable recognizing that Morse seems to have sampled only one location in the area covered in this study. He reported observing black specimens in unspecified locations between Norway, Maine, and Norridgewock [18], indicating that the separation in 1920 was, at most, shorter than the 80 km separating those locations.

Part of the answer may lie in the migratory avian predators that *P. ignipectus* encounters in its Maine habitats. Insectivorous birds migrating to and from the southeastern USA would encounter *P. bicincta* in abundance in many grassy habitats, including cattle pastures. Likewise, birds migrating to and from Mexico and Central America would encounter Neotropical pasture spittlebugs with similar lined color patterns, including the phenotypically similar species *Prosapia simulans*, *Aeneolamia contigua*, and *Aeneolamia albofasciata* [34, 35], which constitute parts of a putative Müllerian mimicry ring in pastures and sugar cane plantations from Mexico to Costa Rica (VT observations).

It is likely that some of the avian predators that encounter *P. ignipectus* in Maine have already learned an aversion to lined color patterns at points south and are predisposed to select in favor of lined color patterns in Maine. However, this in itself does not explain why the lined form has achieved monomorphism in one small geographical area over a protracted period of time. Work with the arctiid moth *Parasemia plantaginis* in Finland suggests that the composition of local avian predator communities can influence the relative fitness of polymorphic aposematic color forms [36] and local populations of the same avian predator can

vary in their response to aposematic prey [37]. This suggests that detailed study of the foraging and migration patterns of Maine insectivorous birds might shed light on *P. ignipectus* color form selection. There are hints in the literature that some Neotropical insectivorous birds specialize in jumping Hemiptera, including Cercopidae [38, 39], though none of the species studied are migratory to Maine.

Independent of the selective factors that might maintain the two distinct aposematic color forms in close proximity, the sharp boundary, apparently without intermediate polymorphic populations, itself poses interesting evolutionary questions. How is it possible to maintain such a sharp boundary for a strong flier in a region with no significant barriers to dispersal? One possibility is that factors other than aposematic selection reinforce the separation of populations. For example, Lis et al. [40] suggest that the endosymbiont bacterium *Wolbachia* may play a role in maintaining genetic barriers at contact zones between mitochondrial linages of the spittlebug *Philaenus spumarius* in Eastern Europe. Likewise, in the absence of definitive genetic evidence to the contrary, it is possible that the lined and unlined Maine populations represent an undetected case of cryptic speciation. If so, similar questions regarding lack of long-term selection for a single color form would still apply, but in the context of two species, not one.

The existence of three Rumford area populations polymorphic for aposematic forms violates the expectation that local populations of aposematic organisms will be monomorphic, but there is ample precedent for polymorphic warning coloration in the Cercopidae. For example, *P. simulans*, an abundant and widespread pasture grass pest in Mexico and Central America, has females polymorphic for a lined form and a dark form [34, 35]. Color polymorphism is also present in populations of the Costa Rican pasture pests *Prosapia plagiata* and *Zulia vilior* [35], the Costa Rican *Heliconia* spittlebug *Mahanarva costaricensis* [41], and the South American sugar cane pest *Mahanarva bipars* [42], among others. Consequently, whether the Rumford area local polymorphism proves to be stable or fleeting, it is consistent with the observation that the Cercopidae as a group are susceptible to color polymorphism [10].

Interestingly, local polymorphisms for aposematic color forms also occur in many other taxa, despite ample theoretical expectation that they should be rare and transient [1, 2, 6, 36, 43–46]. These "almost embarrassingly frequent" occurrences of polymorphism [6, 47] may be due to simultaneous multiple predator selection for warning coloration and rarity [48]; to spatially varying selection pressures in complex Müllerian mimicry systems [6]; to complexities of predator sampling strategy [47]; to balancing selection for warning coloration, sexual signaling, and thermal melanism [2]; or to a mélange of factors [43, 49]. Additionally, it has been suggested that relatively weak defenses [43, 50–52] or defenses that vary in effectiveness against mixed predators [53] might be especially conducive to polymorphism for aposematic forms. This hypothesis may be applicable to spittlebugs as a group, including some species in the Aphrophoridae and Clastopteridae that lack the reflex bleeding characteristic of the Cercopidae (see [54] and Figures 7 and 42–45 in [16]).

The rare cooccurrence of black and lined forms of *P. ignipectus* in populations close to the *bicincta-ignipectus* species boundary raises another set of questions, unlikely to be resolved in the absence of molecular genetic analysis. These populations might reflect introgression through hybridization, analogous to the hybrid zones between parallel color forms of *Heliconius erato* and *H. melpomene* [3]. Alternatively, they could reflect incomplete selection for homogeneity of the black form following an earlier speciation event in which *P. bicincta* gave rise to *P. ignipectus*; genetic reversion to a lined phenotype only recently lost as suggested by Boring [19]; or altered avian selection regimes due to closer proximity to *P. bicincta* populations to the south.

In his initial report of lined Maine spittlebugs, Morse [18] put the question succinctly: "Why the banded form alone should be found at the Norridgewock locality and only there ... at the northern limit of the distribution of the species ... is as yet an unsolved problem." The problem is still unsolved but perhaps now within reach of solution, given recognition that the color forms in question are aposematic and the ongoing development of comprehensive tools for the analysis of selection for warning coloration [1, 6, 44, 47]. Because they involve a single species, without the complicating presence of local Müllerian mimics, Maine *P. ignipectus* populations may present an especially tractable case for analysis of the causes and context of polymorphism for warning coloration.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Corrigendum

Corrigendum to "Intraspecific and Intracolonial Variation in the Profile of Venom Alkaloids and Cuticular Hydrocarbons of the Fire Ant *Solenopsis saevissima* Smith (Hymenoptera: Formicidae)"

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In the article titled "Intraspecific and Intracolonial Variation in the Profile of Venom Alkaloids and Cuticular Hydrocarbons of the Fire Ant *Solenopsis saevissima* Smith (Hymenoptera: Formicidae)" [1], the method description was incomplete and inaccurate. Thus, the last part of the "2.4. GC-MS Analyses" section "The injector temperature was 250°C. Oven temperature was programmed to increase at 12°C/min from 50°C to 330°C, with a final hold time of 1 min" should be corrected to "The injector temperature was 250°C. Oven temperature was programmed to increase at 12°C/min from 50°C to 290°C with a hold pause for 6 minutes, followed by an increase of 30°C/min from 290°C to 320°C followed by a final hold time of 1 min."

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Review Article

Reproductive Interference and Niche Partitioning in Aphidophagous Insects

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The range and quality of prey species differ greatly among closely related species of predators. However, the factors responsible for this diversified niche utilization are unclear. This is because the predation and resource competition do not always prevent species coexistence. In this paper, we present evidence in support of reproductive interference as a driver of niche partitioning, focusing on aphidophagous insect. Firstly, we present closely related generalist and specialist species pairs in aphidophagous lacewings to compare the reproductive interference hypothesis with two other hypotheses that have been proposed to explain niche partitioning in lacewings and sympatric speciation through host race formation and sexual selection. Secondly, we present a case study that shows how reproductive interference can drive niche partitioning in sibling ladybird species. Thirdly, we show that many ladybird genera include species inhabiting the same region but having different food and habitat preferences, raising the possibility that reproductive interference might occur in these groups. Finally, we show that intraguild predation cannot always explain the niche partitioning in aphidophagous insects including hoverflies and parasitoids. On the basis of the evidence presented, we urge that future studies investigating predator communities should take account of the role of reproductive interference.

1. Introduction

In nature, closely related species often occupy niches that diverge with respect to both type and breadth, and such niche divergence is observed not only in herbivores but also in many predators [1–4]. A frequently proposed explanation of niche differentiation is the trade-off hypothesis, according to which adaptations of a species that allow it to exploit one resource decrease its fitness on other resources, and this trade-off leads to different niche utilization by different species [5]. In predators, the main driving force of food specialization may be morphological and behavioral adaptations that enhance a species' prey capture performance against one prey species but simultaneously reduce it against another [6]. However, the trade-off hypothesis is inadequate to explain differences in food choice among some closely related predator species. In particular, some species that possess traits that make them highly efficient foragers specialize in a highly defended prey species, even though they could potentially utilize a wide variety of food items, including less well-defended prey (e.g., [7–9]). For example, large-mouthed marine cottid fish species have been shown in a laboratory setting to have a high feeding performance on both slow-moving prey (e.g., crabs, isopods, and gastropods) and more mobile, and thus more elusive, prey (e.g., fishes and shrimp), but in nature they feed predominantly on elusive prey [10]. Such examples raise the possibility that negative interspecific interaction, rather than a species' own resource-use traits, can restrict its host range in nature.

Examples of negative interspecific interactions that might promote niche partitioning among predators include intraguild predation and parasitism, where species

competing for a shared resource also prey on or parasitize one another [11, 12]. Predation by predators on predators has been widely reported under both laboratory and natural conditions [13, 14]. Moreover, multiple species involved in intraguild predation have been observed to persist in the same locality in field studies (see the "Aphidophagous Guilds"). Therefore, the idea that intraguild predation, which combines predation with resource competition, is sufficient to drive niche partitioning in predators is still controversial. Thus, we would propose that it should be fruitful to consider alternative interspecific interactions that might explain the divergent food choices of carnivorous species.

One reported type of interspecific interaction between closely related predator species is heterospecific mating interactions (e.g., [15–17]). For example, in damselflies *(Nehalennia)*, males frequently attempt to form a tandem pair with a heterospecific female, a behavior that may waste the time and energy of both species even if hybridization does not occur [18]. Moreover, in a molecular study, Fitzpatrick et al. [19] detected genetic introgression between specialist and generalist garter snakes, which is indirect evidence that interspecific copulation occurs in nature. In fact, such reproductive interference is a general mechanism that is applicable to various species assemblages with overlapping mating signals, regardless of trophic level, if at least one species engages in sexual reproduction [20, 21]. However, we think that the potential effect of reproductive interference on the spatial distribution and resource use by predator species has been underestimated, possibly because researchers have focused on mechanisms that are specific to predator-prey systems, such as intraguild predation and strong resource competition [22]. Therefore, we assert that reproductive interference should be given greater consideration in the search for a mechanism to explain ecological specialization in predators.

In this paper, we focus on aphidophagous insects. First, we describe aphidophagous lacewing genera that include generalist and specialist predatory species pairs. In lacewings, it has been hypothesized that sympatric speciation has occurred through host race formation or sexual selection. We argue instead that the observed niche partitioning between closely related species may actually be a consequence of niche diversification through reproductive interference after allopatric speciation. Next, we present as a case study of niche partitioning by reproductive interference our own research on two species of ladybirds *(Harmonia)* in Japan. Then, we consider whether reproductive interference-driven niche partitioning can explain niche partitioning in other predatory ladybirds. Finally, we describe some aphidophagous insect communities in which intraguild predation is known to occur and discuss reproductive interference as a possible mechanism that can drive niche partitioning of intraguild predators. We hope that the arguments made in this paper will encourage researchers to pay more attention to interspecific mating behaviors and their ecological consequences in predator communities.

2. Specialist and Generalist Lacewings in North America

In this section we first examine the possible role of reproductive interference in the ecology of two sibling species (i.e., reproductively isolated species that are nearly identical in their appearance [25]) of the lacewings (Neuroptera: Chrysopidae) in North America, one in genus *Chrysopa* and another in *Chrysoperla*. The *Chrysopa* pair includes a strict specialist that uses a strange woolly aphid, and the *Chrysoperla* pair includes a conifer specialist. Then we compare the reproductive interference model with models of sympatric speciation based on host race formation and sexual selection, which have been considered the main drivers of genetic and habitat diversification in sympatric lacewings.

2.1. Chrysopa and Chrysoperla Species Complexes. *Chrysopa quadripunctata* is a generalist predator that preys upon a wide variety of aphids and other soft-bodied arthropods that live on various plant species, including apple, elm, goldenrod, hickory, maple, oak, and rose. In contrast, *Chrysopa slossonae* is a specialist predator that exclusively utilizes the woolly alder aphid, *Prociphilus tessellatus* [31]. Interestingly, the woolly alder aphid covers its body with secreted wax, which it uses to attract the ants that protect the aphid colonies from predators [31]. The wax may also serve as a physical defense against attacking predators [32]. However, *C. slossonae* larvae exhibit a sophisticated behavior in which they scrape the wax from their prey and attach it to their own back, thus preventing their detection by the attending ants [31]. In addition to this camouflage strategy, the specialized morphology (long legs and a large mandible) of *C. slossonae* larvae may enable them to consume the woolly alder aphid efficiently [33].

We argue that reproductive interference by *C. quadripunctata* may be responsible for the restricted food range of *C. slossonae*, for the following reasons. First, *C. slossonae* larvae can develop on less elusive prey that they do not utilize in nature, such as *Myzus persicae* and *A. pisum* [7, 34]. These observations are evidence that the fundamental niche of *C. slossonae* is wider than its realized niche. Moreover, interspecific copulation and hybridization occur between *C. slossonae* and *C. quadripunctata*, at least under laboratory conditions [34]. Although interspecific pairs of *C. slossonae* and *C. quadripunctata* can produce viable hybrids, the ecological traits of such hybrids show the intermediate values between their parent species [34]. This indicates that hybridization may hamper the local adaptation to each habitat. Therefore, it is possible that *C. slossonae* is forced to specialize on a highly defended prey to avoid heterospecific mating interactions with *C. quadripunctata*, which utilizes various preferred prey species in nature.

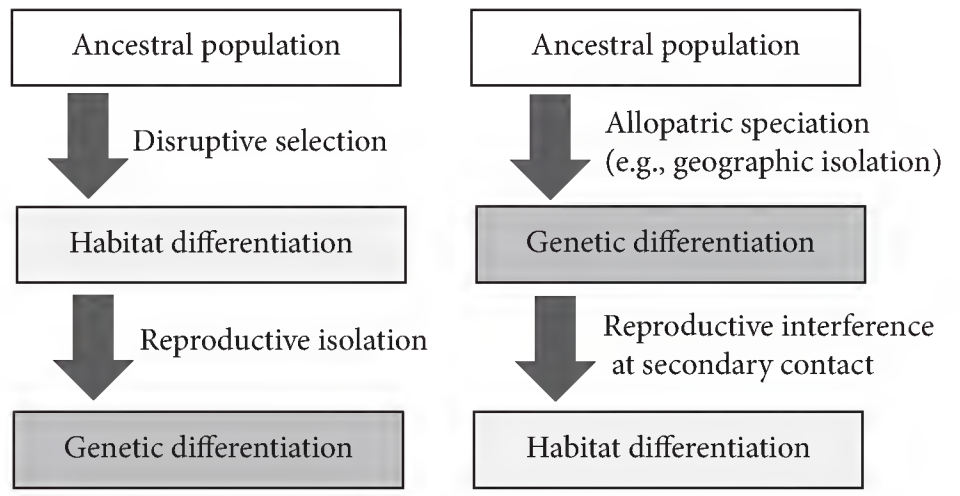
Chrysoperla carnea is a habitat generalist that lives in fields and meadows during the summer and migrates to deciduous trees in autumn. In contrast, its sibling species, *Chrysoperla downesi*, is a habitat specialist, living all year only in coniferous trees, in the region of eastern North America where the two species are sympatric [38]. It can be inferred

that *C. downesi* specializes on conifer-associated prey items, but the information about the variety and quality of prey utilized by these species is still scarce. Each species, however, exhibits phenotypic traits that suit it to its preferred habitat. First, both species display cryptic adult body coloration that is specific to their habitats: *C. carnea* is bright green in spring and summer and reddish brown in autumn and winter, whereas *C. downesi* is dark green all year [39]. Second, each species has life-history traits, such as the critical day length for diapause induction (e.g., [40]), that suit it to its voltinism. *Chrysoperla carnea* can utilize a variety of food sources depending on the season and produces several generations each year, whereas *C. downesi* is restricted to a single generation in the spring, probably because the availability of their prey in coniferous trees is more seasonal. As in the *Chrysopa* species complex, hybridization can occur between *C. carnea* and *C. downesi*, at least under laboratory conditions [39], which suggests that reproductive interference might occur in nature.

At present, the available laboratory findings on interspecific mating behavior are insufficient for the role of reproductive interference in these *Chrysopa* and *Chrysoperla* species to be evaluated. The hybrids were experimentally produced to clarify the existence of postmating reproductive isolation [34, 39] and to examine the genetic basis for the phenotypic traits [39, 40]. However, all possible behavioral mechanisms that can reduce adult reproductive success should be considered in investigations of the population dynamics of these two species pairs. For example, interspecific sexual harassment prior to interspecific copulation can reduce a female's oviposition rate, longevity [41], and mating success [42], which might have population dynamics consequences and lead to subsequent niche separation. Thus, future studies of these sibling lacewing species should not only examine hybrid production but also quantify lifetime female reproductive success to evaluate the possibility that reproductive interference has driven the niche separation.

2.2. Sympatric Speciation through Host Race Formation. The difference in niche width between sympatric lacewings has been attributed to sympatric speciation [43, 44]. Sympatric speciation occurs when ecological adaptations to a different host (habitat) also produce nonrandom mating in a conspecific population, which eventually leads to a phenotypically and genetically divergent subpopulation, or species, even without geographical isolation [45]. In lacewings, courtship and copulation generally take place on plants that also serve as the feeding sites for both the adults and their offspring [46], indicating that adaptation and fidelity to a different habitat by each species may contribute to the development of reproductive isolation.

Tauber et al. [44] have argued that the comparative evidence from this *Chrysopa* species pair demonstrates for the first time a sympatric speciation mechanism in predatory insects. They proposed that a generalist ancestor of *C. slossonae* adopted a specialized foraging behavior and morphology as well as cryptic adult coloration that were well suited to a specific habitat. At the same time, a seasonal



(a) Sympatric speciation by habitat specialization

(b) Character displacement by reproductive interference

FIGURE 1: Two models of ecological specialization and niche differentiation in sibling lacewing species. (a) Sympatric speciation model. Disruptive selection within an ancestral population (i.e., ecological adaptation to different habitats) drives reproductive isolation, which leads to genetic differentiation and, subsequently, complete speciation. (b) Reproductive interference model. Reproductive interference drives habitat differentiation between the two species when secondary contact occurs after allopatric speciation.

reproductive cycle, different from that of the ancestor, became established in the specialized subpopulation. This subpopulation then became reproductively isolated, both spatially and temporally, from the original population, which eventually led to its becoming a new specialist species by sympatric speciation.

Here we compare the two evolutionary scenarios, sympatric speciation and ecological specialization via reproductive interference, that have been proposed to explain the current niche partitioning between sibling lacewing species. The assumed order of ecological adaptation and genetic divergence differs between these two models [47] (Figure 1). In the sympatric speciation model, habitat differentiation is the primary impetus of genetic divergence in an originally uniform population. In this model, therefore, reproductive isolation is a by-product of adaptation to a particular habitat [48] (Figure 1(a)). In contrast, in the ecological specialization through reproductive interference model, genetic divergence (speciation) occurs allopatrically and reproductive interference promotes habitat differentiation when the two already distinct species come into secondary contact [49, 50]. In the reproductive interference mechanism of the second model, therefore, genetic incompatibility between the species is the cause of niche diversification (Figure 1(b)). Thus, the two models predict similar ecological consequences, namely, utilization of different habitats by two genetically close species, which prevents interspecific sexual interactions [47]. Accordingly, it is difficult to judge which model is applicable to a particular study system by examining current phenotypic traits and fitness.

The model of sympatric speciation in *Chrysopa* and *Chrysoperla* lacewings has some as yet unresolved problems. Most importantly, the generalist predator has been observed to visit the habitat on which its sibling species specializes, even though that habitat is less suitable for the generalist

species. For example, *C. quadripunctata* is sometimes found on alder trees, where *C. slossonae* preys on the woolly alder aphid [34]. In addition, *C. carnea* visits not only meadows and deciduous trees but also coniferous trees, where *C. downesi* is found year-round [39]. This flexible behavior on the part of generalist predators may allow them to add a less suitable prey to their menu when the availability of more suitable prey is limited for some reason (e.g., [51]). Moreover, Henry [38] pointed out that the breeding periods of the sibling lacewing species greatly overlap, even though C. A. Tauber and M. J. Tauber [39] considered the seasonal mismatch to be an important mechanism of temporal reproductive isolation. Therefore, it is possible that observed niche overlap in space and time might be insufficient for sympatric genetic divergence to have occurred in the *Chrysopa* and *Chrysoperla* systems. Nevertheless, because genetic divergence sometimes occurs despite copulation and gene flow between taxa [52–54], the apparent incomplete habitat isolation does not rule out sympatric speciation in these lacewings. At this time, therefore, it is not possible to rule out any particular view of speciation in the lacewing system.

2.3. Speciation through Sexual Selection. Henry [38] proposed that divergence in the courtship song, rather than habitat specialization, might be a primary driver of speciation in *Chrysoperla* lacewing species, whether allopatric or sympatric. In *Chrysoperla*, males produce substrate-transmitted calls on plants by vibrating their abdomen, and females respond to a potential partner by reciprocal signaling according to their own mating preference [55]. Henry [38] proposed that, based on the diversified and complex songs among close relatives, differentiation of courtship signals among populations should create ethological barriers to hybridization, thus accelerating the rate at which complete speciation can be achieved.

However, there is at least one problem with speciation through sexual selection. *Chrysoperla* females sometimes respond to heterospecific mating calls [55], which indicates that negative interspecific mating interactions can occur. In fact, under laboratory conditions copulation and hybridization can occur among populations (species) having different courtship songs [39]. These findings suggest that an ethological barrier alone might not ensure premating reproductive isolation.

This weak point of the speciation through sexual selection model (i.e., inevitable heterospecific mating interactions), however, supports the reproductive interference model. In general, the maintenance of incomplete species recognition abilities can be interpreted as a consequence of adaptive decision-making in reproductive animals. When the signals of high-quality conspecifics resemble those of heterospecifics, a trade-off is likely to exist between species recognition and mate-quality recognition within a species [56]. In this situation, strict species discrimination skill is unlikely to be maintained because individuals may also lose opportunities for acquiring high-quality conspecific mates. As a result, promiscuous mating occurs between the species. By this logic, reproductive interference is likely to be common in sibling species (because their mating signals are likely to be similar), and the reproductive interference might drive niche partitioning.

3. A Case Study in *Harmonia*

3.1. A Brief History of Studies of Harmonia Sibling Species. The multicolored Asian ladybird *Harmonia axyridis* is a common aphidophagous predator originally distributed in Russia, China, the Korean Peninsula, and Japan [24]. Because of its extreme polymorphism with regard to elytral color, *H. axyridis* has been extensively used for genetic studies. Theodosius Dobzhansky famously discovered geographic variation in the frequency of elytral color morphs across Siberia and East Asia [57]. Subsequently, extensive surveys established that elytral color morph frequencies change along a geographic (latitudinal) cline in Japan, where red (non-melanistic) morphs at higher latitudes are gradually displaced by black (melanistic) morphs as latitude decreases [58]. Then, Hosino, who worked at an agricultural high school in Sanage, central Japan, discovered that elytral color morph frequencies were slightly different between *Harmonia* ladybirds collected from Japanese red pine *(Pinus densiflora)* and those collected from agricultural crops such as wheat, pear, and peach [59]. In addition, he reported that a ridge at the tip of the elytron, which is a genetic character exhibited by approximately 40% of *H. axyridis* individuals in the Sanage population, was seldom observed in individuals collected from pine trees [59]. For a few decades, Japanese entomologists were unable to account for these strange patterns.

Eventually, Hiroyuki Sasaji demonstrated the existence of a cryptic *Harmonia* species. He did so by experimentally demonstrating that a clear postmating reproductive barrier existed between *H. axyridis* individuals and those found on pine trees, even though they were observed to copulate together and produce eggs [60]. He also showed that larval coloration was distinct between these groups of individuals [61] (Figure 2), even though the adults were difficult to distinguish by their external and genital morphology [60], and, furthermore, he showed that the characteristic ridge at the tip of the elytra of *H. axyridis* adults never emerged in the pine-associated individuals [60]. On the basis of these findings, he concluded that the pine-associated individuals belonged to a cryptic species that could be clearly distinguished from *H. axyridis* by its postreproductive isolation and some phenotypic traits.

However, when Sasaji proposed denominating the new, pine-associated cryptic species, he discovered that Takizawa [62], at the dawn of insect taxonomy in Japan, had already described a species of ladybird collected from a pine tree and had named it *Ptychanatis yedoensis* (*Ptychanatis* is a currently unused synonym for genus *Harmonia*). Takizawa's illustration of a larva of his new species (Figure 2(c)) clearly shows the larval characteristics of the cryptic species "discovered" by Sasaji. Sasaji [63], therefore, identified the cryptic species as *Harmonia yedoensis* Takizawa. In addition, deeply moved by Takizawa's insight, Sasaji proposed *Kurisaki-tento* as the Japanese name of this species, because Takizawa had adopted

(a) (b) (c)

FIGURE 2: (a) A final instar larva of *H. axyridis* on a *Spiraea thunbergii* leaf. (b) A final instar larva of *H. yedoensis* on Japanese red pine, *Pinus densiflora*. (c) Illustration of a final instar larva of *H. yedoensis* by Kurisaki [23].

(a) (b)

FIGURE 3: (a) A giant pine aphid, *Cinara pini*, on Japanese red pine, *Pinus densiflora*. (b) *Harmonia yedoensis* hatchling capturing a *C. pini* individual, observed in a laboratory experiment. Because *C. pini* has long legs and is highly mobile, *H. yedoensis* larvae are often unsuccessful in their attempts to capture this aphid.

the name "Kurisaki" when he became a Buddhist priest, and *tento* is the Japanese word for ladybird.

3.2. Host Specialization in H. yedoensis. The existence of these two species, *H. yedoensis* and *H. axyridis*, living in close proximity raises some questions; namely, what determines their habitat ranges? And how did two such phylogenetically close species with such a close morphological resemblance, at least in adults, come to occupy dramatically different niches? *Harmonia axyridis* is a truly generalist predator found in many different habitats, including scrub, orchards, grasslands, and coniferous and deciduous woodlands (e.g., [51]). In contrast, *H. yedoensis* is a specialist in central Japan that breeds exclusively on pine trees, where its larvae are regarded to prey mainly on the giant pine aphid *(Cinara pini)* [24] (Figure 3(a)). Because aphid colonies are spatially heterogeneous and temporally variable in terms of both quality and quantity, a generalist strategy that allows the ladybird to utilize multiple food patches has obvious advantages for maximizing lifetime fitness [51], as it does for other generalist insect species (e.g., [64–66]). Therefore, how to explain host specialization, a strategy that appears to forego potentially available resources, has been an interesting problem in ecology and evolutionary biology.

It is clear from field surveys that *H. yedoensis* is much less abundant than *H. axyridis*. One result of this situation is that

much more effort is required to collect enough *H. yedoensis* individuals to carry out research projects, compared with that required for *H. axyridis*. The rarity of *H. yedoensis* might be at least partly due to the low abundance of its prey item on pine trees. Although the yellow egg batches of *H. yedoensis* are easy to locate on dead or live pine tree needles, it is hard to locate nearby colonies of the giant pine aphid, which are usually very small (S. Noriyuki personal observation), unlike those of other aphid species, which tend to congregate on young shoots of their host plants. Syunsuke Shimamoto, a junior high school student studying the ecology of *H. yedoensis*, once remarked to one of the authors (S. Noriyuki), "I wonder why *H. yedoensis* larvae stay where there is so little food to eat, while *H. axyridis* larvae are on plants with lots of aphids." We, too, ask this simple question.

Not only are giant pine aphids rare, they are also so mobile that ladybirds seem to have difficulty capturing them. In contrast to most arboreal aphids, which walk very slowly at best, the giant pine aphid has long legs and can walk fast (Figure 3(b)). We therefore experimentally evaluated the prey capture performance of *H. yedoensis* and *H. axyridis* hatchlings against the giant pine aphid and other aphid species [9]. As predicted, it was hard for *H. axyridis* hatchlings to capture the giant pine aphids. Interestingly, however, although *H. yedoensis* apparently specializes on the giant pine aphid, this species is not the easiest prey for *H. yedoensis* hatchlings to catch. Rather, we found that *H. yedoensis* hatchlings could capture other aphid species more easily, even though they never encounter them or prey upon them in nature. These results led us to hypothesize that *H. yedoensis* mothers deliberately choose a host that is less suitable for the prey capture activities of their offspring even though other suitable hosts are available in the region.

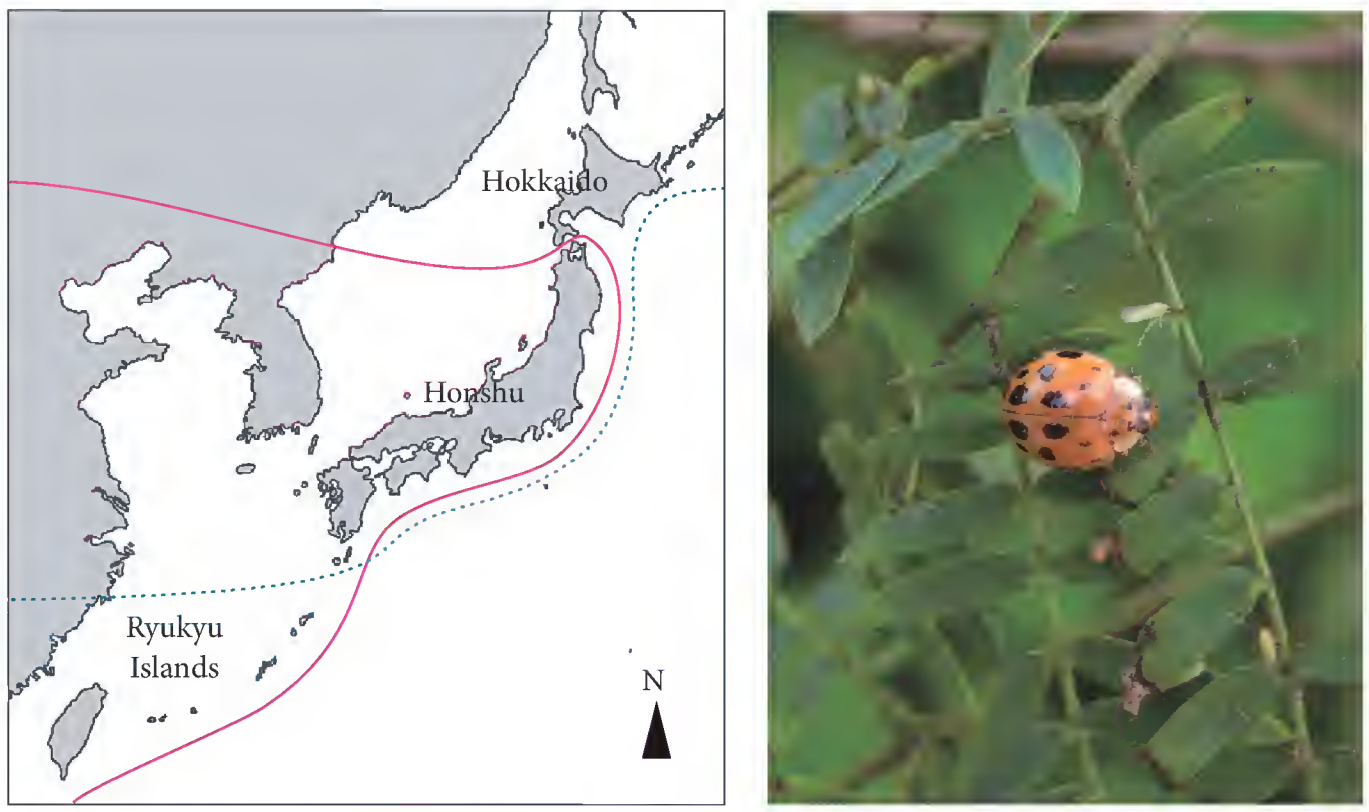
Harmonia yedoensis mothers help their offspring cope with their elusive aphid prey by investing more maternal resources in each hatchling. In aphidophagous ladybirds, the hatch rate of an egg batch is often less than 100% and the newly hatched larvae consume the unhatched sibling eggs. These unhatched eggs are not just an unavoidable developmental side effect; they are an adaptive maternal strategy known as trophic egg provisioning, because ladybird mothers are able to control the proportion of unhatched eggs in a clutch according to food availability [67]. In fact, trophic egg provisioning is more intense in *H. yedoensis* than in *H. axyridis* [26, 28]. Moreover, artificial manipulation of the trophic egg number provided to each hatchling has revealed that trophic egg consumption enhances the prey capture performance of *H. yedoensis* hatchlings against the giant pine aphid [9]. In addition, *H. yedoensis* eggs are significantly larger than *H. axyridis* eggs [26, 28]. Consequently, the amount of maternal resource invested per offspring is much higher in *H. yedoensis* than in *H. axyridis*. Importantly, however, the number of offspring that a mother can produce in her lifetime is lower in *H. yedoensis* than in *H. axyridis*, because of the trade-off between offspring size and number in the similar-sized species [26]. Therefore, *H. yedoensis* mothers apparently sacrifice reproductive success in order to supply more resources to each of her larvae, which are obligated to prey on the elusive aphid.

Furthermore, we found by experiment that the giant pine aphid is nutritionally less suitable for the larval development of ladybirds. We froze some aphids, to exclude the effects of prey mobility on larval performance, and then fed them to larvae of *H. yedoensis* and of *H. axyridis* to evaluate their intrinsic suitability as food [27]. The result showed that in both ladybird species larval developmental performance was lower in larvae fed the giant pine aphid than in those fed the other prey species. Although we have not yet examined the proximate mechanism for the lower developmental performance, secondary compounds derived from the pine trees and stored in the aphid's body (see [68, 69]), or simply an insufficient nutrient content, might be responsible.

Taken together, these findings show that the giant pine aphid is poor prey for both *H. yedoensis* and *H. axyridis* larvae with respect to abundance, capture difficulty, and its intrinsic suitability as food. Therefore, we conclude that food quality per se does not determine the food range of *H. yedoensis*. In contrast to the rarity and niche specialization of *H. yedoensis*, *H. axyridis* is abundant and utilizes various preferred prey species. These differences suggest that negative interactions between *H. yedoensis* and *H. axyridis* might greatly influence the food utilization of *H. yedoensis* in nature.

3.3. Reproductive Interference between the Two Harmonia Species. The results summarized in Section 3.2 led us to hypothesize that reproductive interference by *H. axyridis* against *H. yedoensis* is responsible for the specialization on less suitable prey by *H. yedoensis*. When *H. yedoensis* was first identified as a cryptic *Harmonia* species, it was already known that although interspecific copulation occurred, a postmating reproductive barrier existed between *H. yedoensis* and *H. axyridis* [60, 70]. Sasaji [60] also showed that the male genitalia were very similar between the two species with respect to both size and morphology, so there was no structural barrier to interspecific copulation. To examine the possible influence of reproductive interference on niche partitioning, it was necessary to quantify the effects of interspecific mating behaviors on reproductive success, which might affect the population dynamics of the two species.

Therefore, we performed some laboratory experiments to examine interspecific mating behaviors and their subsequent effect on reproductive success [29]. Both *H. yedoensis* and *H. axyridis* males attempted to mate with both conspecific and heterospecific females, and these attempts often resulted in interspecific copulation lasting a few hours or more. Females of both species can lay eggs even after interspecific copulation, but no viable offspring hatched. However, *H. axyridis* males tended to choose conspecific over heterospecific females, whereas *H. yedoensis* males promiscuously copulated with both conspecific and heterospecific females. We are unable to account for this difference in discrimination skill between *H. yedoensis* and *H. axyridis*, although chemical signals based on cuticular hydrocarbons or visual cues related to body size and color might be important for the interspecific communications. But, probably owing to this difference in discrimination skill, most *H. yedoensis* individuals failed to copulate with conspecifics, especially when the density of *H.*



(a) (b)

FIGURE 4: (a) Natural distribution of *H. yedoensis* (red solid line) and *H. axyridis* (blue dotted line) around the Japanese archipelago (modified from Sasaji [24]). The two species are sympatric in central Japan (Honshu), whereas only *H. yedoensis* is found in the Ryukyu Islands and only *H. axyridis* in Hokkaido. (b) *Harmonia yedoensis* on a lead tree, *Leucaena leucocephala*, on Miyako Island, southern Ryukyus. In areas where *H. axyridis* does not occur, *H. yedoensis* adopts a generalist strategy.

axyridis was high in the experimental arena. By contrast, most *H. axyridis* individuals successfully copulated with conspecifics regardless of whether *H. yedoensis* was present [29]. Here, it is important to note that the fitness reduction at the mating stage is asymmetric between these two species; that is, *H. yedoensis* is inferior to *H. axyridis* in terms of reproductive interference.

These results support the hypothesis that reproductive interference from *H. axyridis* has led to host specialization in *H. yedoensis*. In nature, *H. yedoensis* might suffer from a considerable fitness reduction if they utilize less elusive aphids in a patch occupied by *H. axyridis*. By specializing to the habitat of the giant pine aphid, which is especially elusive prey for *H. axyridis*, *H. yedoensis* can mitigate the cost of reproductive interference. Therefore, we argue that *H. yedoensis* mothers are obligated to choose lower quality prey for offspring performance where the ranges of these sibling species overlap.

Biogeographical distribution patterns also suggest that reproductive interference plays a nonnegligible role in determining the food range of *H. yedoensis*. In the Ryukyu Islands, southern Japan, where *H. axyridis* is not found (Figure 4), *H. yedoensis* often visits various deciduous tree species [71, 72] (S. Noriyuki, personal observation). In contrast, on Hokkaido Island, northern Japan, where *H. yedoensis* does not occur, *H. axyridis* is a generalist predator, just as it is in regions of central Japan where the two species are sympatric. These patterns suggest that *H. axyridis* utilizes various preferred prey species without regard to the presence of *H. yedoensis*, whereas the negative impact of reproductive interference of *H. axyridis* on *H. yedoensis* forces the latter to become a specialist predator on less preferred prey where the two species coexist.

Comparison of *Harmonia* (ladybirds) and *Chrysopa* (lacewings) (Table 1) reveals many similarities in the relationships between these sympatric sibling species pairs. Both specialist species can potentially develop on a variety of food sources that are in nature utilized exclusively by their generalist counterpart. Moreover, the specialist species have morphological, behavioral, and physiological adaptations that enable them to handle their less suitable prey. Furthermore, interspecific copulation can occur between members of each pair, at least under laboratory conditions. Given these observations, it would be interesting to explore whether the mechanism of ecological specialization/niche differentiation is the same in these two aphidophagous taxa.

Of course, alternative interspecific interactions, not necessarily mutually exclusive with reproductive interference, must also be considered as possible mechanisms of the ecological specialization of *H. yedoensis*. In particular, intraguild predation may drive host specialization in *H. yedoensis*, because *H. axyridis* is known to be a strong intraguild predator against other ladybird species. However, the results of a laboratory experiment suggest that at the larval stage *H. axyridis* might not be dominant over *H. yedoensis* as an intraguild predator [73]. It is probable that the body size of the individual larvae, rather than their species identity, mainly determines the winner in interactions between these two predatory larvae, because the final instar larvae of both species feed on earlier instar larvae of the other species when they are put together in a Petri dish [73]. We think it is likely that intraguild predation and reproductive interference jointly contribute to niche partitioning between these two ladybird species, because both mechanisms can destabilize species coexistence locally. However, the direction of the food specialization, with *H. yedoensis* feeding on

Table 1: Comparison of the niche, life-history traits, and interspecific mating interactions in sibling species between *Harmonia* ladybirds and *Chrysopa* lacewings.

	Harmonia ladybirds		*Chrysopa* lacewings	
	H. axyridis (generalist)	*H. yedoensis* (specialist)	*C. quadripunctata* (generalist)	*C. slossonae* (specialist)
Niche				
Habitat	Various deciduous trees including pine trees	Pine trees	Various deciduous trees including alder trees	Alder trees
Prey	Various aphid species	The giant pine aphid	Various aphid species	The woolly alder aphid (with attended ants)
Alternative prey	Various aphid species and artificial diet	Various aphid species and artificial diet	Various aphid species	Various aphid species
Life-history traits				
Adult body size	Similar		Small	Large
Egg size	Small	Large	Small	Large
Rate of oviposition	High	Low	High	Low
Sibling cannibalism within clutch	Low	High	NA	
Hatchling head size	Small	Large	Small	Large
Larval leg length	Short	Long	Short	Long
Larval behavior	Low walking ability	High walking ability	Camouflage	Camouflage with wax
Seasonality	Bivoltine (central Japan)	Univoltine	Bivoltine and a partial third generation per year	Univoltine
Critical photoperiod for diapause induction	NA		Short	Long
Interspecific reproduction				
Interspecific mating attempt	Yes		Yes	
Interspecific copulation	Yes		Yes	
Viable hybrid offspring	No		Yes	
Reproductive interference	Superior	Inferior	NA	

Information on *Harmonia* is from Sasaji [24], Osawa and Ohashi [26], Noriyuki and Osawa [27], and Noriyuki et al. [9, 28–30]; information on *Chrysopa* is from Albuquerque et al. [7], Eisner et al. [31], Milbrath et al. [32], Tauber et al. [33], and C. A. Tauber and M. J. Tauber [34].

more elusive and *H. axyridis* feeding on less elusive prey, might be determined by the asymmetry of the reproductive interference. Nevertheless, the combined effect of predation and competition on individual fitness and species coexistence is predicted by mathematical models to produce complex outcomes [74, 75]. We therefore think that it would be very interesting to see how the incorporation of reproductive interference in a trophic module along with intraguild predation would alter population dynamics and community structure.

Now, about 100 years after Takizawa first described a new *Harmonia* ladybird species and several decades since its rediscovery by Sasaji, it is becoming clear that the mechanism of ecological specialization and generalization in *H. yedoensis* and *H. axyridis* involves negative interspecific interactions. This case study illustrates how the integration of taxonomy, ecology, and evolution can deepen our understanding of natural history and the great principles that underpin biological communities.

4. Other Specialist Ladybirds

Reproductive interference may have led to niche partitioning not only in *Harmonia* species but also in predatory ladybirds belonging to other genera. Therefore, we picked congeneric species pairs known to show different food (habitat) ranges or types in the same geographical region and examined the incidence of interspecific mating interactions in these congeneric pairs. Then we investigated conifer- (pine) associated specialist ladybirds in Britain and Japan to assess the generality of our hypothesis that reproductive interference can lead to niche partitioning in closely related species.

4.1. Niche Partitioning between Congeneric Ladybird Species Pairs. To examine niche partitioning in other ladybirds, we selected genera that contain at least two species with different food (habitat) types in the same region of Britain or Japan (Table 2). In both these countries, the natural history of ladybirds has been well studied by professional

Table 2: Comparison of niche utilization among sympatrically distributed congeneric ladybird species of the United Kingdom (UK) and Japan.

Genus	Species	Food, host plants, and habitats
UK		
Adalia	*A. bipunctata*	Various herbaceous and arboreal habitats
	A. decempunctata	Various arboreal habitats, but more specialized than *A. bipunctata*
Chilocorus	*C. renipustulatus*	Coccids; broad-leaved deciduous trees
	C. bipustulatus	Coccids; *Calluna*, Leyland cypress, and other trees; heathland
Coccidula	*C. rufa*	Reeds, reed-mace, rushes, and wetland grasses
	C. scutellata	Reeds, reed-mace, and rushes
Coccinella	*C. undecimpunctata*	Aphids; herbaceous habitats, especially in coastal areas
	C. quinquepunctata	Aphids; low-growing herbaceous plants such as nettle, thistles, bitter-cress, and angelica; unstable river shingle
	C. septempunctata	A variety of aphid species on an extensive range of low-growing herbaceous host plants; habitats including agroecosystem, grassland, heathland, and coniferous and deciduous woodland
	C. magnifica	Ant-attended aphids; Scots pine and other plants close to ant nests of genus *Formica*
	C. hieroglyphica	Larvae of the heather leaf beetle *Lochmaea suturalis* and the heather aphid *Aphis callunae*
Nephus	*N. bisignatus*	Low-growing vegetation in coastal regions
	N. quadrimaculatus	Coniferous and deciduous woodlands
	N. redtenbacheri	Various low-growing vegetation in both inland and coastal regions
Scymnus	*S. nigrinus*	Needleleaf conifers
	S. frontalis	Low-growing vegetation in dry habitats and on coastal dunes
	S. femoralis	Low-growing vegetation on well-drained soils
	S. schmidti	Various types of low-growing vegetation
	S. haemorrhoidalis	Low-growing vegetation and small shrubs, particularly in damp areas
	S. auritus	Oak trees
	S. limbatus	Willow, sallow, and poplar trees
	S. suturalis	Needleleaf conifers, occasionally deciduous trees
	S. interruptus	Pseudococcids and diaspidids in diverse habitats
Rhyzobius	*R. chrysomeloides*	Pine trees, deciduous trees, and ivy
	R. litura	Low-growing vegetation, especially grasses and thistles
	R. lophanthae	Coccids and diaspidids on trees
Japan		
Calvia	*C. quindecimguttata*	Reeds
	C. muiri	Various habitats, especially bamboo grasses
Chilocorus	*C. kuwanae*	Coccids such as *Pseudaulacaspis pentagona*
	C. rubidus	*Kermococcus* coccids on plum, cherry, chestnut, and oak
	C. mikado	*Quercus gilva*
Harmonia	*H. axyridis*	Various habitats, mainly arboreal
	H. yedoensis	Pine trees
Oenopia	*O. scalaris*	Pine trees
	O. hirayamai	Various arboreal habitats
Pseudoscymnus	*P. sylvaticus*	Inside galls of *Tuberocephalus sasakii* on cherry and of *Ceratovacuna nekoashi* on storax
	P. pilicrepus	Eusocial aphid *Colophina arma* on the subshrub *Clematis stans*
Scymnus	*S. posticalis*	Various herbaceous and arboreal habitats
	S. yamato	Wetland, mainly on reeds
	S. babai	Wetland
	S. ohtai	Wetland
	S. hoffmanni	Wetland
	S. nakaikemensis	Wetland
	S. otohime	Chestnut; prey is *Moritziella castaneivora,* which infects chestnut cases

Information on UK ladybirds is from Roy et al. [35] and Majerus [36]. Information on Japanese ladybirds is from Sasaji [24] and Shiyake [37].

and amateur entomologists. Because, in this paper, our focus is on aphidophagous insects, we exclude phytophagous and mycophagous ladybirds from the list in the table, but this exclusion does not mean that reproductive interference might not be important in these functional groups [47]. We identified numerous closely related species in various ladybird genera that exhibited niche partitioning (Table 2). For example, *Pseudoscymnus sylvaticus* mothers lay their eggs on galls of certain aphid species (*Tuberocephalus sasakii*, which forms galls on cherry tree leaves, or *Ceratovacuna nekoashi*, which forms galls on fruits of Japanese snowbell, *Styrax japonica*), and the hatched larvae forage exclusively on the aphids in the gall. In contrast, a sympatric congener, *Pseudoscymnus pilicrepus*, utilizes the eusocial aphid *Colophina arma* on the subshrub *Clematis stans* [24]. At present, the drivers of this niche partitioning in regionally coexisting predatory ladybirds are not well understood. Here, we focus on *Adalia* and *Coccinella* species pairs, because each of these genera contains generalist and specialist species pairs distributed sympatrically in Britain and other parts of Europe and considerable information is available about their ecology.

Adalia bipunctata is one of the most common ladybird species in Europe and it occupies a great variety of habitats, including woodlands, scrub, and grassland. By contrast, its sibling species, *Adalia decempunctata*, is more habitat-specific; it is found mainly on deciduous trees and in hedgerows [76–78]. Because in *A. decempunctata* larvae can develop and forage on various aphid species [79] and mothers produce more eggs when fed on an aphid that is not regularly used in the field [80], this habitat restriction cannot be explained solely by food suitability. Moreover, Sloggett and Majerus [78] have shown that the prevalence of parasitoids is similar between *A. bipunctata* and *A. decempunctata*, suggesting that the habitat specialization of the latter is not driven by a need for a habitat with low predation risk (i.e., an enemy-free space [81]).

Under laboratory conditions, interspecific copulation occurs between *A. bipunctata* and *A. decempunctata*, and the resulting sterile hybrids occasionally develop into adults [82]. Thus, interspecific mating interactions should reduce their reproductive success when the two species occupy the same local patch. During fieldwork in the Netherlands, Brakefield [76] importantly observed that the principal spring mating habitats of *A. bipunctata* were shrubs, whereas he found almost all mating *A. decempunctata* on trees. This use of different mating habitats suggests that past negative mating interactions might have caused small-spatial-scale divergence of reproductive sites in these species. It would be interesting to explore this possibility empirically in the future.

Coccinella septempunctata is also very common in gardens and agroecosystems, but it is also found in heathland, scrub, and coniferous and deciduous woodlands. In contrast, each of the four other *Coccinella* species in Britain specializes in a different niche (Table 2). In particular, *Coccinella magnifica* is a myrmecophile that lives only near ant nests and it preys on aphids that are attended by the ants [83]. Obviously, foraging on ant-attended aphids is costly for *C. magnifica* adults and larvae, because the ants often attack the foraging ladybirds [84, 85], suggesting that the ant attendance itself cannot explain the niche differentiation between *C. septempunctata* and *C. magnifica*. Importantly, *C. magnifica* can be successfully reared in the laboratory from adult to adult by feeding nonattended aphid species such as *Acyrthosiphon pisum* and *Aphis fabae* (i.e., alternative foods). Thus, its potential dietary breadth appears to be similar to that of its generalist congener *C. septempunctata* [83]. Even though interspecific mating behaviors have not been thoroughly studied in these two ladybird species, the adult body size and color pattern are very similar between them. It might be illuminating, therefore, to consider interspecific mating interactions in future investigations of niche partitioning among *Coccinella* species. For example, to test the hypothesis that reproductive interference prevents a species from occupying its fundamental niche and restricts it to a narrower realized niche, experiments should be performed to examine whether reproductive interference occurs in these species and whether the cost of the interaction is incurred more by *C. magnifica* than by *C. septempunctata*.

4.2. Conifer Specialists. It is well known that some species of predatory ladybirds utilize specialized habitats on pine trees and other conifers (Table 3) and that this specific specialization has independently evolved several times in the ladybird lineage [86]. Not all conifer-specialist species coexist in the same region with a congeneric relative (Table 3), which suggests that, assuming that reproductive interference is mainly likely to occur among phylogenetically close species, these specializations might have occurred through mechanisms other than reproductive interference. Some conifer specialists, however, do have sympatrically distributed generalist congeners (Table 3). In Britain, the habitats of *Scymnus nigrinus* and *Scymnus suturalis* are restricted to conifer trees, whereas a congener, *Scymnus schmidti*, utilizes a wide range of habitat types [35]. A similar pattern has been observed in Japan in specialist and generalist species belonging to genus *Oenopia* [24]. Therefore, we can hypothesize that, as in *Harmonia* species in Japan, this specialization to conifers might have been driven by reproductive interference from sympatric congeners. To test this hypothesis, it will be necessary to quantify the cost of interspecific mating interactions as well as the quality of the conifer habitat with respect to larval and adult development. It would be interesting to know if reproductive interference has caused some species to specialize to conifers more than once in the ladybird phylogeny.

5. Aphidophagous Guilds

In this section we consider intraguild predation as an alternative mechanism of the niche partitioning of aphidophagous insects. Multiple species of aphidophagous insect predators, as well as parasitoids that attack aphids, often coexist despite the occurrence of intraguild predation. Osawa [87], for example, studied aphidophagous ladybirds and hoverflies preying on eight species of aphids infecting seven species of deciduous trees in the Botanical Garden of Kyoto University, central Japan. He collected five aphidophagous ladybird species and

Table 3: Conifer-associated specialist ladybird species in the UK and Japan.

Species	Detail	Sympatric congeners
UK		
Exochomus quadripustulatus	Coccids on needleleaf conifers, sallows, and willows	NA[1]
Myrrha octodecimguttata	Aphids on Scots pine	NA
Sospita (Myzia) oblongoguttata	Aphids on Scots pine	NA
Harmonia quadripunctata	Aphids on needleleaf conifers	*H. axyridis*[2]
Anatis ocellata	Aphids on needleleaf conifers, particularly pines	NA
Scymnus nigrinus	Scots pine	*S. schmidti* and other species[3]
Scymnus suturalis	Needleleaf conifers, particularly Scots pine, but occasionally deciduous trees	*S. schmidti* and other species
Japan		
Harmonia yedoensis	Aphids on Japanese red pine and Japanese black pine	*H. axyridis*
Sospita (Myzia) oblongoguttata	Pine trees	NA
Oenopia scalaris	Pine trees	*O. hirayamai*

Information on UK ladybirds was adapted from Roy et al. [35] and Majerus [36]; information on Japanese ladybirds was adapted from Sasaji [24] and Shiyake [37]. [1]NA indicates that no congeneric species is distributed in the same region; [2]*H. axyridis* is an exotic species in the UK; [3]refer to Table 2 for other *Scymnus* species.

eight hoverfly species (see also [88]). Notably, both field [89] and laboratory studies [90, 91] have shown that four of the collected ladybird species (except *Scymnus posticalis*) engage in intraguild predation. *Harmonia axyridis*, in particular, is an intraguild predator that preys predominantly on other ladybird species [89, 92, 93]. Nevertheless, the abundance of *H. axyridis* is not negatively correlated with the abundance of the other four ladybird species in this area [88]. Thus, some mechanism must exist that allows these ladybird species to coexist in the same patch.

Similarly, in hoverfly communities generalist predators often use many of the same prey species [94, 95]. Moreover, aphidophagous ladybirds and hoverflies often coexist where their distributions overlap [88, 89, 96], even though predatory ladybirds often attack hoverflies with which they compete for the shared aphid resource (reviewed by [97]). Thus, in aphidophagous communities, predation and resource depletion do not necessarily result in strict niche partitioning.

If parasitoids that attack aphids are assumed to belong to the same guild as aphidophagous insects [13], then aphidophagous communities become even more species-rich and complex. In this case, intraguild predation includes predators such as ladybirds that feed on parasitized aphids (e.g., aphids that have been mummified), as well as different parasitoid species that, when parasitizing the same aphid, attack each other. Despite this complex food web structure, multiple parasitoid species are known to utilize the same aphid colonies simultaneously. For example, Müller et al. [98] reported that, in Silwood Park, southern England, some species of aphids were infected with multiple parasitoid species. Moreover, Osawa [87] identified various primary and secondary parasitoids on aphids on which both predatory ladybirds and hoverflies intensively foraged. These observations suggest that some factor must exist that permits the coexistence of multiple species in aphidophagous communities with multiple and complex prey-predator links.

The conditions that allow the stable coexistence of a predator and its prey, together with a shared prey species, have been extensively analyzed by mathematical models, as well as by some empirical studies. One classic model [12] predicts that stable coexistence should be possible if a trade-off exists between resource consumption and predation ability, but only under a limited range of environmental conditions. In addition, mechanisms such as a temporary refuges for prey [99], optimal predator foraging behavior [100, 101], and intense intraspecific interactions [102] have been proposed to allow species coexistence despite the occurrence of intraguild predation. These mechanisms likely explain the seemingly paradoxical robust persistence of many natural intraguild predation systems.

The local coexistence of phylogenetically closely related species, however, may still be inhibited. In Japan, two sibling aphidophagous ladybirds, *Propylea japonica* and *Propylea quatuordecimpunctata*, are distributed parapatrically; the former lives at low elevation and the latter at high elevation, but within a narrow zone they coexist [24]. In Britain, *Coccinella septempunctata* utilizes a wide range of prey types and habitats, whereas *Coccinella hieroglyphica* is mainly found on heather, where it forages on the heather aphid, *Aphis callunae*, and on larvae of the heather leaf beetle, *Lochmaea suturalis* [35]. It has often been demonstrated that in both animal and plant communities local assemblages tend to be composed of phylogenetically distant species (e.g., [103–105]), probably owing to exclusive interactions among closely related species. Therefore, to understand the observed spatial distributions of aphidophagous insects, in addition to intraguild predation, some factor that is tightly connected to phylogenetic closeness should be considered.

Interspecific mating behavior is more likely to occur among phylogenetically close species because they often share similar mating signals [56, 106]. The resulting, often costly mating interactions can lead to local species extinction,

which in turn can cause diversification of niche use and resource-use traits [49]. In contrast, phylogenetically distant species, which generally have distinctive, easily discriminated mating signals, focus their mating efforts on high-quality conspecifics even when their ecological niches largely overlap. As a result, distantly related species can stably coexist within a patch [49]. These observations suggest that reproductive interference might be a mechanism of niche diversification in closely related species.

6. Conclusion

We have shown that the effect of reproductive interference on local population dynamics has probably been underestimated, in contrast to the intraguild predation mechanism, which is specific to the predator community. Therefore, to evaluate the validity and generality of the reproductive interference mechanism, more empirical studies, and extensions of published studies, of various predator species are needed. We have suggested some possible approaches earlier in this paper. Moreover, the relative importance and synergistic effects of predation, exploitative competition, and reproductive interference are largely unknown. Because these mechanisms of species exclusion are not mutually exclusive, the impact of each individually, and of their interactions, on niche partitioning needs to be quantified. A theoretical research priority is the incorporation of reproductive interference into the intraguild predation model to examine how it changes the population dynamics. Together, such empirical and theoretical efforts will enable us to evaluate whether niche partitioning in predators is explicable mainly by reproductive interference, a mechanism that occurs in several functional groups, including herbivores [42, 50] and plants [107], or by a specific mechanism that is unique to carnivores such as intraguild predation.

At present, it cannot be conclusively decided whether the observed niche partitioning between phylogenetically close species has been caused by sympatric speciation or reproductive interference after allopatric speciation, because it is difficult to distinguish between these two evolutionary scenarios by examining current phenotypic traits and fitness. Thus, a fruitful approach might be to identify the locality of the origin of the species by appropriate techniques such as phylogeography and genomics. Sympatric speciation has been frequently assumed to explain niche partitioning among phylogenetically similar relatives such as cryptic species complexes [108], but compelling evidence that two sympatrically distributed species (races) have diverged is still lacking [109]. A theoretical basis now exists for ecological character displacement via reproductive interference [49]. Incorporation of reproductive interference into existing models should make it possible to design more rigorous tests and to further refine the models and thus improve their skills in reconstructing the natural world.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Species Composition and Visiting Frequencies of Flower Visitors of *Chromolaena odorata* in a Dry Zone Forest Patch of Sri Lanka

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Plant-animal interaction has been a major theme in ecology as it has helped ecologists to rule out different patterns they observed in the surrounding environments. *Chromolaena odorata* is another plant species that is studied extensively as it has become a major troublesome weed in many parts of the tropics. But, handful of studies are available on pollination of this invasive plant species in dry forests and its function as a pollinator sink in these environments. The current study was carried out in a dry zone secondary forest patch in North-Central Sri Lanka to assess the diversity, abundance, and pollination strength of flower visitors associated with the *C. odorata*. The results suggest that the diversity of Hymenoptera and Lepidoptera is higher than the other insect orders that visited *C. odorata*, but all species exerted equal pollination strength on the plant. The attraction of large numbers of insects is concluded to as one of the factors that contribute to the reproductive success of *C. odorata* in dry zone forests.

1. Introduction

Transfer of pollen grains to the stigma of the flower gynoecium (Pollination) is the crucial event in sexual reproduction process of flowering plants that ensures their long last survival [1–3]. Therefore, self-incompatible flowering plants in the world heavily rely on animals for their pollination [1, 3, 4]. Thus, plants benefit from these mutualistic interactions to ensure effective cross pollination and to maintain viable population in their habitats.

Disturbances that alter pollinator communities in habitats where self-incompatibale flowering plants are abundant may reduce the pollination services. Additionally, loss or low abundance of pollinating partners will lead ecologically specialized plants to edge of local extinctions. These distrupting factors can be (1) human alterations to environments reducing pollinator abundance (agriculture, pesticides effect); (2) the effect of invasive, ineffective pollinator species that exclude effective native pollinators from floral resources (pollinator competition); and (3) introduced exotic flowering plants with higer attractability than native flowering plants [5]. However, most of the pollinator communities consist of higher number of invertebrate generalists and few number of specialists who visit flower for floral resources [6–8]. Thus, tropical pollinator networks have developed an immunity against the decline of specialized pollinators but it gives no gurantee that the fidelity between interaction partners will be kept after alterations to the system occur.

Importance of pollination has been well documented. But, handful of studies are available which explore insect visitors in exotic plants while studies on controlling invasive plants are being largely available. According to our understanding, studies on plant-insect mutualism in certain weeds are extremely important as they might reveal several underlining causes to their inherent invasiveness [9] in the habitats they are invading. Among different adaptive characteristics seen in exotic invasive plant species, competing with native species for resources available in particular environment including pollinators is commonly observed [10]. This may bring a detrimental effect on native existing mutualisms. Growing as considerably large stands and mass flowering with higher amount of reward for pollinators, these exotic

species attract pollen dependent insects that visit other native flowering plants [9]. This abundance in exotic floral resources is known to alter the spatial distribution of floral resources and by that they compete for pollinators with native self-incompatible flowering plants [5]. One of the resons that the exotic plants gets more dispersed is having high success in pollination and thereby high seed production to extend their dispersal range [11]. In contrast, several authors argue that abundance of exotic plants with high amount of floral resourses is beneficial to neighboring native plants because they increase pollinators in the area by acting as a sink [12].

A native of Central and South America, *Chromolaena odorata* (L.) King and Robinson, the Siam weed has spread throughout the tropical and subtropical areas of the world and is now a major weed in Central and Western Africa, South and Southeast Asia [13]. This neotropical weed is being flagged as one of the most troublesome weeds in many humid tropical countries [14]. Being perennial and having the ability to withstand dry climate, fires [15, 16], and stressed environmental conditions [17, 18] *C. odorata* has become more abundant weed in the above regions while showing potential extension of its distribution.

This study was conducted to assess pollinators that visit widely dispersed *C. odorata* in tropical secondary dry forest patch in North-Central Sri Lanka. Visitation frequencies were utilized as a surrogate to measure the importance of flowering plant to potential insect pollinators as a forage source.

2. Materials and Methods

2.1. Chromolaena odorata. C. odorata formerly known as *Eupatorium odoratum* L. is a weedy pioneering shrub [13, 16, 19]. This invasive plant has spread over South Asian region from mid-nineteenth century and currently stated as the most troublesome weed in the region [13, 14, 19].

C. odorata is a scrambling perennial shrub, with straight, pithy, and brittle stems, usually grown up to 2-3 m in height. But with support from other vegetation and environmental conditions it can reach up to 5–10 m in height [15, 19]. Capitula are located in panicles at the ends of the branches and are with only disk florets. Each floral head usually consisted of 21 to 28 tiny tubular florets. These flower heads are about 10 mm long and 3 mm wide, pale pink or pale mauve in color, bisexual, and actinomorphic. The ligule is tubular with five teeth at the tip. Five epipetalous stamens arise from the base of the corolla; filaments are free but anthers are united, syngenesious. The anthers are dithecous, and the pollen grains are very small, round, and ornamented. The styles of florets are stretched beyond anthers and its branches are receptive to pollen deposited by flower visitors [9]. In the end of the first growth season, the plants undergo sexual reproduction cycle producing approximately 8400 seeds/m^2 [20] and reproduction becomes majorly apomictic in later periods of the life cycle. Seeds are mainly wind-dispersed and they also exhibit the tendancy to stick to fur, feather, and clothes.

2.2. Study Site. The study was conducted in Mihintale Sanctuary where dense vegetation of *C. odorata* is found. The site is located in the dry zone of Sri Lanka between 8° 18′–8° 23′ N and 80° 27′–80° 35′ E and extends over 999.6 ha (2470 acres) area. Extent of the sanctuary has been defined in the gazette number 8370 of Sri Lankan government in 1938, yet no proper demarcated boundaries are documented [21].

Mihintale sanctuary receives an annual rainfall between 1,000–1500 mm/year from Northeast monsoon (November–January) and intermonsoons (March-April, September-October). The temperature fluctuates between 19°C and 38°C throughout the year with mean annual temperature of the study area being 27.3°C. From February to September is the dry period with August being the warmest month of the year. The Southwest monsoon winds blow across Mihintale between May and September creating higher evaporation rate.

Mihintale sanctuary consists of different kinds of habitats; dry zone semievergreen undisturbed secondary forest patches, semievergreen disturbed secondary forests, scrublands, aquatic habitats, and highly degraded forest patches. *C. odorata* was observed as dense stands in around these habitats as well as the open forest margins.

2.3. Data Collection. A field survey was conducted to document potential pollinators in *C. odorata* shrubs in the area of 0.5 km^2 semievergreen forest patch from the end of August 2014 to January 2015 which coincided with one flowering season of the weed [9, 16, 22]. Five (5) *C. odorata* inhabiting sites were selected to study flower visitors in Mihinthale Sanctuary. Vegetation density was calculated using the quadrate sampling method. Altogether, thirty (30) 5 m × 5 m quadrats were layed randomly over stands in five study sites and number of *C. odorata* plants were counted in each quadrat. Counts were averaged and the density of *C. odorata* was calculated. Flowering density for 1 m^2 was calculated by counting and averaging the number of flower heads fell within randomly layed another thirty (30) 1 m × 1 m quadrates on *C. odorata* strands [23].

Direct observation method was followed to encounter potential insect visitors to *C. odorata* stands in sunny days with gentle breeze [24, 25]. Rainy days with heavy winds were deliberately avoided as pollinators are disturbed by these unsuitable climatic conditions. Prior to insect observations, flowers were observed carefully to confirm the readiness of flowers for pollination. Mature flowers from stands were sampled and observed to confirm that pollination has not been carried out [4, 26]. This was easily determined by observing whether the stamens were disturbed and/or pollens were robbed by flower visitors. Fifteen (15) 1 m × 1 m quadrats were selected randomly to be observed for insect visits. Observations and sampling insects were done from 0700 to 1800 for 7 days during the flowering season with the assistance of two observers. Visitation frequencies of each insect were noted during 30-minute period per hour. Insect samplings were done carefully using an insect net causing minimum as possible disturbance to the visiting insect communities. Therefore, five hours per each day was effectively utilized for insect survey. Interactions were confirmed if an insect touches anther or stigma of the flower [27] and visits were recorded irrespective to previous visits of the insect to the

same plant. 30-minute settling period was given to the flower strands after each 30-minute capturing session to reduce the magnitude of disturbance created by the observers when capturing insects. Captured insect assemblages were taken to the laboratory to identify using available keys. [28]. Some specimens were identified to the genus by referring to the Invertebrate Systematic and Diversity Facility (ISDF) of the Department of Zoology, University of Peradeniya. At the laboratory, insects were checked for adhering pollens under the optical microscope to ensure whether the insects act as a potential pollinator or not. The insects that do not carry pollens on their bodies were excluded from the counts. The insect collections were deposited with the laboratory insect collection of the Rajarata University of Sri Lanka for further studies.

2.4. Data Analysis. The data gathered during the field survey was tabulated as insects in rows and visitation frequencies in columns. This contingency table was used to calculate different indices for describing the topography of the observed flower-insect interaction system.

Richness of insect visitor community was calculated using an abundance-based coverage estimator and richness of each insect order was calculated using Margalef's Index (M). Diversity of flower visiting insects was calculated using Maximum Likelihood Estimator of Shannon Diversity Index [29]. Diversity indices were calculated using the program SPADE developed by Chao and Shen [30].

Mean visitation frequencies were calculated for each insect observed in all 30 quadrats. The strength of interaction (T), which is indicative of the impact that each insect visitor exerts on flowers, was calculated following methods in Vazquez et al. [31]. Interaction strengths were checked for significant difference among flower visitor families and among flower visitor orders using a one-way ANOVA with residual analysis for normality and homoscedasticity of variance. All statistical analysis on interaction strength and visualization was done using MINITAB version16 (Minitab Inc., Coventry, UK).

3. Results

Flowering occurred from October to December. Mean *C. odorata* vegetation density was 51.6 ± 5.14 while mean flowering density was 312.53 ± 8.32 across five sites. A total of 2271 flower visitations were recorded during 35 hours within 7 days of observation to *C. odorata* strands. Seventy-seven (77) insect flower visitors were recorded belonging to five major insect orders and twenty-five families (Table 1). Species richness of the *C. odorata* flower visitor community was 77.5± 0.80 and species diversity was 4.183 ± 0.02.

Lepidopteran, Hymenopteran, and Dipteran diversity was higher than the diversity of Hemipterans and Coleopterans (Table 2). Altogether 32 Lepidopterans belonging to five families, 30 Hymenopterans belonging to eight families, 10 Dipterans in seven families, three Hemipterans in three families, and two Coleopterans in two families were identified as potential pollinators in the study system.

The interaction frequency of the pollinator species is highly correlated with the species strength (i.e., impact exerted on plant by its interacting partners) [31]. Thus, the most frequent flower visitors were thought to have a potentially high contribution for pollination of flowers and by that increasing their reproductive fitness or have higher amount of the pollinator reward from the flower. Orders Hymenoptera (0.014 ± 0.001), Diptera (0.013 ± 0.001), and Lepidoptera (0.012 ± 0.001) showed higher interaction strengths, respectively, while the order Coleoptera (0.009 ± 0.001) had the least strength.

When comparing interaction strengths of flower visitors, family Apidae and Halictidae in the order Hymenoptera, family Syrphidae and Calliphoridae in order Diptera, and family Nymphalidae and Lycaenidae in the order Lepidoptera exhibited higher values in interaction strength scale. However, we did not detect any statistically significant differences between means of interaction strengths of flower visitor families as determined by one-way ANOVA [F (24, 52) = 0.69, $p \geq 0.841$]. These results suggest that each insect visitor has equal importance in pollinating this invasive weed in dry forests.

Though there is no significant difference in pollinator strengths among insect visitors, top contributors were identified by ranking them in descending order using their impact exerted on the plant. *Apis cerana, Apis dorsata, Ceratina binghami, Lasioglossum amblypygus, Antepipora* sp. of order Hymenoptera, *Episyrphus nectarinus* of the order Diptera and *Cepora nerissa, Junonia hierta, Melanitis leda,* and *Castallius rosimon* of order Lepidoptera exhibited higher strength in pollination.

Observers identified two types of insect visitors exhibiting two different types of behaviors, namely, foragers on flowers and hovers. All insects who had shown high species strength over flowers were observed always on flowers. They all were observed to forage on closely arranged capitula of *C. odorata* passively loading sticky pollen grains on insects body. Thus, they were expected to facilitate passive transport of pollen grains more than sudden visitors who visit flower only for second or two.

4. Discussion

C. odorata is a weedy plant attracting a rich diversity of insects. Being available during major flowering season of the dry forest as well as the dry seasons, it provides an important source of pollen, nectar, and foraging grounds to insects who share the same habitat that they grow in. In general, insect communities associated with cosmopolitan invasive weeds differ from one habitat to another depending on availability of particular species and essentially on availability of other nectar and pollen sources [5]. Thus, the interactions are product of relative abundance of plant and animal components and environmental alterations. But, some authors argue that, irrespective of the species living in the habitat, exotic plant species reconstruct the plant-animal interactions by altering the resource availability to pollen or nectar gatherers [5]. In that context researchers can deduct that being an exotic plant in dry zone secondary forests *C. odorata* shares the

Table 1: List of flower visitors in different orders and families observed in *C. odorata* stands in Mihintale Sanctuary and the frequency of visits. Rates presented are population means per 1 × 1 m flower strands per hour (n = 15, 35 hours).

Order	Family	Species	Frequency	SD (±)
Hymenoptera	Apidae	*Amegilla comberi*	2.33	1.53
		Apis cerana	22.67	1.15
		Apis dorsata	19.00	2.00
		Apis florea	14.00	2.65
		Braunsapis sp.	7.33	4.16
		Ceratina binghami	15.67	1.53
		Thyreus insignis	5.00	1.00
		Trigona iridipennis	9.00	1.00
		Xylocopa ruficornis	11.33	0.58
		Xylocopa tenuiscapa	4.00	1.00
	Halictidae	*Curvinomia iridiscens*	13.00	1.00
		Hoplonomia westwoodi	12.00	1.73
		Lasioglossum amblypygus	18.33	2.08
		Leuconomia sp.	12.33	1.53
		Pachynomia sp.	8.00	1.00
	Megachilidae	*Euaspis edentata*	12.00	1.00
		Lithurgus atratus	7.00	1.00
		Megachile lanata	12.67	2.08
		Megachile vigilans	4.33	0.58
	Chrysididae	*Chrysis oculata*	4.33	0.58
		Stilbum cyanurum splendidum	10.00	1.00
	Eumenidae	*Antepipona* sp.	15.00	1.00
		Delta emarginatum	7.00	1.00
		Subancistrocerus sichelii	2.00	1.00
	Sphecidae	*Ammophila atripes*	2.67	0.58
		Sp. *1 (H1)*	13.67	1.53
		Sp. *2 (H2)*	12.67	2.08
		Sphex sp.	7.00	1.00
	Scelionidae	*Scolia jurinei* Sauss	3.00	1.00
	Vespidae	Sp. *1 (H8)*	3.67	2.52
Coleoptera	Meloidae	*Mylabris phalerata*	8.67	0.58
	Coccinellidae	Coccinellidae	9.00	4.00
Diptera	Syrphidae	*Episyrphus nectarinus*	16.67	0.58
	Asilidae	*Cophinopoda chinensis*	13.67	2.52
		Sp. *2 (D1)*	6.33	2.08
	Ulidiidae	Sp. *1 (D2)*	5.33	2.52
	Rhagionidae	Sp. *1 (D3)*	4.33	0.58
	Bombyliidae	Sp. *1 (D4)*	8.00	1.00
	Calliphoridae	Sp. *1 (D5)*	10.00	2.65
		Sp. *2 (D6)*	14.00	2.14
		Sp. *3 (D7)*	12.33	1.12
	Tephritidae	Sp. *1 (D8)*	7.33	1.53
Hemiptera	Reduviidae	*Ectomocoris cordigera*	9.33	0.58
	Pyrrhocoridae	Sp. *1 (He1)*	6.33	2.08
	Scutelleridae	*Rhynchium brunneum*	12.00	2.00

Table 1: Continued.

Order	Family	Species	Frequency	SD (±)
		Pachliopta hector, Crimson Rose	7.33	1.53
	Papilionidae	*Papilio demoleus,* Lime butterfly	7.33	0.58
		Papilio polymnestor, Blue Mormon	3.67	2.08
		Papilio polytes, Common Mormon	14.00	4.36
		Appias albina, Common Albatros	12.33	1.53
		Appeals Galen, Lesser Albatros	3.33	1.53
		Appias libythea, Stripped Albatros	2.67	2.08
	Pieridae	*Catopsilia pyranthe,* Mottled Emigrant	8.33	2.52
		Cepora nerissa, Common Gull	17.33	2.89
		Delias eucharis, Common Jezebel	4.67	1.15
		Eurema blanda, Three-Spot Grass Yellow	10.33	3.21
		Eurema hecabe, Common Grass Yellow	2.33	1.53
		Acraea violae, Tawny Coster	8.00	1.00
		Cirrochroa thais, Tamil Yeoman	14.00	1.00
		Danaus genutia, Common Tiger	11.00	1.00
Lepidoptera		*Euploea core,* Common Crow	14.67	2.08
	Nymphalidae	*Junonia hierta,* Yellow Pansy	17.33	1.15
		Junonia iphita, Chocolate Soldier	14.33	1.53
		Melanitis leda, Common Evening Brown	21.00	2.65
		Neptis hylas, Common Sailer	5.67	2.08
		Parantica aglea, Glassy Tiger	5.67	1.53
		Ypthima ceylonica, White Four Ring	7.67	1.15
		Castalius rosimon, Common Pierrot	15.00	3.61
		Catochrysops strabo, Forget-me-not	3.00	1.00
	Lycaenidae	*Jamides celeno,* Common Cerulean	8.33	0.58
		Pachliopta hector, Crimson Rose	8.00	4.36
		Rapala manea, Slate Flash	12.00	1.00
		Ampittia dioscorides, Bush Hopper	5.00	1.00
		Badamia exclamationis, Brown Awl	7.33	3.06
	Hesperiidae	*Iambrix salsala,* Chestnut Bob	8.33	1.53
		Spialia galba, Indian Skipper	4.67	2.08
		Telicota colon, Pale Palm Dart	5.33	2.52

Table 2: Diversity and richness of flower visitor orders in *C. odorata* stands in Mihintale Sanctuary (MLE: Maximum Likelihood Estimator of Shannon Weiner Diversity Index; M: Margalef's Index for measuring species richness).

Order	Number of species	Diversity (MLE)	Richness (M)
Coleoptera	2	0.679 ± 0.023	0.351 ± 0.025
Diptera	10	2.227 ± 0.012	1.963 ± 0.022
Hemiptera	3	1.049 ± 0.053	0.602 ± 0.004
Hymenoptera	30	3.259 ± 0.049	5.113 ± 0.058
Lepidoptera	32	3.267 ± 0.050	5.469 ± 0.062

same strength to alter topography of native interaction webs in its associated habitats. Thus, the current study bears an importance in determining the relative importance of the plant to the insect community and the strength it exerts on potential pollinators residing in the habitat. Also, being a plant with higher number of associated potential pollinators, *C. odorata* exhibits its ability to act as pollinator sink in the growing environment.

Shihan and Kabir [32] argue that the attraction of the Butterflies in higher level may be due to presence of hexose sugars and amino acid rich nectar in the nectaries available in flowers. The flower bears minute amount of nectar more than many other flowers used by nectar gatherers. But, bees tend to forage on *C. odorata* as much as on any other nectar plant [33–36]. The reason may be the availability of large quantity of pollens and nectar collectively in one flower stand. Therefore, this weedy plant has been used as an important honey plant in commercial apiculture all over the world [33, 35, 36]. By looking at our insect visitor checklist compiled for dry zone forests shown above it is concluded that wild bees are also attracted to the plant as much as the domesticated bees such as *Apis cerana*, *Apis dorsata,* or *Trigona iridipennis.*

In addition to the nutrients and the reward for the pollinators, the shape of the flowers was also observed to have well adapted to facilitate the visitors. Floral heads create a fine

landing stage for many flower visitors and closely arranged flowers in the stands facilitate movement of insects from flower to flower easily or efficiently.

The current study does not suggest very strong interaction between flower visitors and the plant *C. odorata*. However, the top potential contributors were identified, including butterflies and adding bees and flies to the global checklist of interacting species with *C. odorata*. Since the dry forests generally experience a scarcity of flowering plants during the dry spell and the heavy rains, generalist insect foragers may have to use whatever floral resources available in the system to ensure their survival and continuation of generations. Therefore, almost equal visitation frequencies and interaction strengths can occur due to this dependence of the floral visitors on the available resources. Accordingly, this plant can be viewed as a refuge for insects when it is flowering in invaded habitats, where no other plants are available to give out pollen and/or nectar in the habitat.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

D. G. R. M. M. Kaushalya Rathnayake and W. M. G. Asanga S. T. B. Wijetunga contributed equally to this work.

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