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Blow Flies Visiting Decaying Alligators: Is Succession Synchronous or Asynchronous?

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Succession patterns of adult blow flies (Diptera: Calliphoridae) on decaying alligators were investigated in Mobile (Ala, USA) during August 2002. The most abundant blow fly species visiting the carcasses were *Chrysomya rufifacies* (Macquart), *Cochliomyia macellaria* (Fabricus), *Chrysomya megacephala* (Fabricus), *Phormia regina* (Meigen), and *Lucilia coeruleiviridis* (Macquart). *Lucilia coeruleiviridis* was collected more often during the early stages of decomposition, followed by *Chrysomya* spp., *Cochliomyia macellaria*, and *Phormia regina* in the later stages. *Lucilia coeruleiviridis* was the only synchronous blow fly on the three carcasses; other blow fly species exhibited only site-specific synchrony. Using dichotomous correlations and analyses of variance, we demonstrated that blow fly-community succession was asynchronous among three alligators; however, Monte Carlo simulations indicate that there was some degree of synchrony between the carcasses.

1. Introduction

Blow flies (Diptera; Calliphoridae) are ubiquitous insects during the early stages of animal decay and their larvae are important in estimating the time since death or the postmortem interval (PMI) of a carcass [1]. Larval age of the earliest carrion-arriving blow fly species can be estimated based on data developed from controlled carrion studies [2, 3]. Faunal composition (succession data) of carrion can be predicted for a given area under specific conditions and the composition compared to baseline data obtained from an animal model [4–8].

Insect succession on carrion has been examined in detail in the southeastern United States. Studies have been performed on decaying dogs, *Canis lupus* L., in Tennessee [9]; pigs, *Sus scrofa* L., in South Carolina [10] and Florida [11]; humans, *Homo sapiens* L., in Tennessee [12]; rats, *Rattus rattus* L., in South Carolina [13]; and a variety of vertebrate species in North Carolina [14], Mississippi [15], and Louisiana [16]. There have been few published reports on the subject from Alabama and Georgia, however, are needed for better understanding of blow fly ecology associated with carrion.

Variation associated with blow fly succession on carcasses placed in the same habitat at the same time has not been tested. Hence, this raises the question of whether carcasses placed simultaneously in the same habitat decompose in the same manner and whether all carcasses experience the same blow fly-succession pattern. To test these hypotheses, we simultaneously placed three carcasses in the field and compared the succession of blow flies on each carcass. Specifically, we tested if the pattern or synchrony of calliphorid succession varied among carcasses.

2. Materials and Methods

2.1. Study Area. Sites were located in an evergreen woodlot on the campus of the University of South Alabama, inside the city limits of Mobile, Alabama. The woodlot was dominated by a mixture of pines (loblolly, *Pinus taeda* L., and longleaf,

Pinus palustris Miller) and hardwoods typical of forests occurring on upland areas associated with sandy loam soils. The canopy was mostly closed with two of three sites in this forest having greater than 75% cover; site C had less than 50% canopy cover. Oaks present included sand, *Quercus geminata* Small; southern red, *Quercus falcata* Michaux; turkey, *Quercus laevis* Walter; and water, *Quercus nigra* L. Other hardwoods included American witch-hazel, *Hamamelis virginiana* L.; flowering dogwood, *Cornus florida* L.; red maple, *Acer rubrum* L.; and southern magnolia, *Magnolia grandiflora* L. The magnolia and maple extended up to the bottom of the pine canopy and form a patchy subcanopy. Shrubs in the woodlot included American holly, *Ilex opaca* Aiton; several azalea species, *Rhododendron* spp.; devilwood, *Osmanthus americana* (L.); swamp titi, *Cyrilla racemiflora* L.; large gallberry, *Ilex coriacea* (Pursh); and sparkleberry, *Vaccinium arboreum* Marshall. The floor was sparse with occasional patches of herbaceous growth occurring in small openings. Herbaceous species were dominated by downy danthonia, *Danthonia sericea* Nuttall; fourleaf yam, *Dioscorea villosa* L.; flowering spurge, *Euphorbia corollata* L.; greater tickseed, *Coreopsis major* Walter; longleaf woodoats, *Chasmanthium sessiliflorum* (Poiret); a sedge, undetermined; St. Andrew's cross, *Hypericum hypericoides* (L.); strawberry bush, *Euonymus americanus* L.; and wood spurge, *Euphorbia commutatus* Engelmann. Vines were abundant at each site and included Carolina jasmine, *Gelsemium sempervirens* (L.); catbrier, *Smilax bona-nox* L.; cat greenbrier, *Smilax glauca* Walter; crossvine, *Bignonia capreolata* (L.); and muscadine, *Vitis rotundifolia* Michaux. This woodlot is typical of forests that have established on former mesic to dry-mesic longleaf pine sites following removal of longleaf pine.

2.2. Sampling Protocol.

The American alligator, *Alligator mississippiensis* Daudin, was chosen as the model carcass because they were readily available as fresh-frozen (frozen since May 2002) specimens and relatively little is known about blow fly succession on these animals [16, 17]. Specimens used were accidentally trapped during turtle surveys in the Mobile-Tensaw delta in southwestern Alabama. Dead alligators were sealed in black garbage bags at collection time and frozen at $-20°C$ until needed. Approximately 24 hours before the beginning of the study, the carcasses were placed in a walk in refrigerator at $4°C$ to thaw slowly. Alligator sizes were as follows: site A: 1.65 m, 17.9 kg; site B: 1.68 m, 20.0 kg; and site C: 1.78 m, 24.3 kg. Each alligator was placed in a stainless-steel wire cage (1.8 × 0.35 × 0.25 m; mesh size = 2.5 × 2.5 cm) to prevent carcass disturbance by vertebrate scavengers and cage placed in the woodlot 5 August 2002 at 1000 hours (i.e., day zero). For purposes of this study, calliphorid collection was ceased on 15 August 2002. Cages were arranged along a single transect, 50 m apart.

The decomposition of each alligator was divided into stages following those of Reed [9] and Johnson [18]. The beginning and end of these stages were difficult to discern and we only report approximate time intervals of the stages. It is important to note that these stages are part of a continuum and not categorical; they are used as reference points to compare the physical decomposition of the carcasses and are considered arbitrary in terms of blow fly succession [10, 19, 20].

Sticky fly-paper was used to collect adult blow flies arriving at the carcasses. Two strips of sticky fly paper (120 cm × 4 cm) were placed on top of each cage at 1000 hours daily. After 24 hours, the fly paper was removed and placed in a labeled container with 95% ethanol and new fly paper replaced on cage. Blow flies were later removed from the sticky paper, identified, and returned to alcohol-labeled vials. To supplement sticky-paper collections, aerial netting was performed over the cages (5 minutes per cage). Blow flies collected by aerial netting were killed in the field using a collecting jar laced with ethyl acetate, placed in labeled vials, and later pinned in the laboratory. Identifications were made according to Hall [21], Hall and Townsend [22], Dear [23], and Whitworth [24].

Blow fly larvae were collected daily from different areas on each carcass and the surrounding ground. Larvae were placed in plastic containers and transported back to the laboratory. One-half of the larvae from each carcass were preserved by boiling in water and then placing them in Kahle's solution [25]. The remaining larvae were reared to adults using the following procedure. Larvae ($N = 3–5$ per container) were placed on a small piece of raw calf liver (approximately 10 g) and then wrapped in moist paper towel. A 3 cm layer of vermiculite was added to 150 mL clear-plastic containers; larvae and liver, wrapped in paper towel, were placed on top of the vermiculite. Pieces of cardboard, furnished with small holes for air circulation, were used to cover containers. Containers were held at room temperature (i.e., 22–24°C) with a light: dark regime of 12:12 hours. Containers were inspected twice daily for the presence of adult blow flies.

As the condition of some flies from the sticky-paper were unsuitable for identification, only adult flies that contained all relevant taxonomic characters were included in analyses. It was assumed that damaged specimens would occur in roughly equal proportions among blow fly species. Reared larvae were used to confirm the identity of sticky-paper collected adult blow flies. *Lucilia cluvia* (Walker), *Lucilia eximia* (Wiedemann), and *Lucilia sericata* (Meigen) were collected as adults on the sticky paper and were not reared from larvae found on the carcasses. Voucher specimens have been deposited in the University of South Alabama's Arthropod Depository.

2.3. Statistical Analyses.

All statistical tests were considered significant at $P < .05$, and the experiment-wise rate was adjusted for each correlation to maintain a family error rate of $P = .05$. For each treatment, an experiment-wise adjustment of P-values was made to preserve a family error rate of $P = .05$. For each species of blow fly, a dichotomous (present/absent) correlation was used to determine the degree of temporal association among each site. Hence, for each species, three correlations were calculated, that is, site A versus site B, site A versus site C, and site B versus site

Table 1: Blow fly succession on site A's decaying alligator, Mobile, Ala, USA (August 2002).

Blow fly species	Daily abundance[a,b] (decomposition stage)									
	1 Fresh	2 Bloat	3 Active	4	5	6	7	8	9	10
Chrysomya rufifacies	+	−	+	+	+++	++	+++	++++	++	+
Cochliomyia macellaria	−	−	+	++	+++	++	++	++	+	−
Chrysomya megacephala	−	−	+	++	++	+++	++	++	+	−
Phormia regina	−	−	−	+	++	++	+	−	+	−
Lucilia coeruleiviridis	+	+	+	+	−	−	−	−	−	−

[a] "−" = 0 adults collected, "+" 1–5, "++" = 6–15, "+++" = 16–25, "++++" > 26.

[b] Day 1 represents the first 24 hours of the study; this 24-hour period began at hour zero (i.e., the time of placement of the carcass in the field at 1000 hours on 5 August 2002) till 1000 hours the next morning on 6 August 2002.

C. As these correlations were special cases of the Pearson-product-moment-correlation coefficient, a z test was used to determine significance [26]. To determine if the relative abundance of each species of blow fly collected on the fly paper differed among sites, an analysis of variance (ANOVA) was used, with number of flies for each species as the response variable, site as the main effect, and day as the block (random variable). For significant main effects, differences among means were determined using the Tukey multiple comparison procedure [27]. All data was normalized before statistical tests.

A Monte Carlo approach was used to examine the similarity of community succession among the three sites used in this study. The intent here was to determine if combined-species occurrence for all species of blow flies, among all sites, occurred at a frequency different from that expected by a random model. Combined species co-occurrence (i.e., the number of times a species occurs on the same day at any pair of sites, summed for all species in the analyses) at a frequency greater than that expected by a random model would indicate predictable community succession among sites [28]. In contrast to correlation or ANOVA analyses, all species were considered simultaneously in this procedure. Our observed test statistic was the total number of co-occurrences for all species. For example, if a particular species occurred at sites A and B on the same five days, site B and C on the same four days, and sites A and C on the same six days, then the total number of observed co-occurrences among sites for that species would be 15. Adding the number of co-occurrences for all five species of blow flies considered in our study produces the observed test statistic.

The Monte Carlo procedure allows the probability distribution for the test statistic (in our case, the number of times a species occurs on the same day at any pair of sites, summed for all species in the analyses) to be generated while permitting the incorporation of relevant biological constraints into the model used to generate the test-statistic distribution [29, 30]. The constraint used in generating our test statistic distribution was that the frequency of each species' occurrence, at each site, was equal to the observed frequency for that species at that site. The test statistic distribution was generated using 1000 Monte Carlo simulations [29] and the observed number of total co-occurrences was

then compared with the generated distribution, and if the P-value of the observed co-occurrence was low (i.e., $P < .05$), then the observation was judged to be significant.

3. Results

Climatological data was obtained from a weather station located 6.5 km from the study sites. The mean daily temperature during this study (5–15 August 2002) was $26.8 \pm 0.5°C$ with a mean daily high of $31.2 \pm 0.9°C$ and a mean daily low of $22.8 \pm 0.5°C$. Rainfall was limited to a total of 7.5 cm during the study, most (5.2 cm) of the precipitation occurred during the first 24 hours.

Eight species of Calliphoridae were identified from decaying alligators during this study; *Chrysomya rufifacies* (Macquart) ($N = 253$, 31.6% of total 806 blow flies), *Cochliomyia macellaria* (Fabricius) ($N = 216$, 27.0%), *Chrysomya megacephala* (Fabricius) ($N = 148$, 18.5%), *Phormia regina* (Meigen) ($N = 100$, 12.5%), *Lucilia coeruleiviridis* ($N = 80$, 10%), *Lucilia cluvia* ($N = 4$, 0.5%), *Lucilia eximia* ($N = 4$, 0.5%), and *Lucilia sericata* ($N = 1$, 0.1%). Daily relative abundances of adult blow flies at each carcass and stage of decomposition are presented in Tables 1, 2, and 3. The fresh stage began at time zero and ended approximately at 24 hours. *Lucilia coeruleiviridis* was the most prevalent blow fly active about the carcasses in the first 24 hours, ovipositing in and around the eyes, mouth, and nostrils. *Lucilia coeruleiviridis* were noted ovipositing on the carcasses within 15 minutes of being placed in field. At the time of this oviposition, it was not raining.

The bloat stage lasted 1–3 days depending on the site. By the end of this stage, at sites A and B, larval masses enveloped the head and limb-torso junctions. At site C, decomposition was slower with maggot masses restricted to the head. The majority of flies visiting the carcasses during this stage were *Lucilia coeruleiviridis* (Tables 1–3). Blow flies encountered in very low numbers during this stage were *Lucilia cluvia* ($N = 4$, site B, day 2), *Lucilia eximia* ($N = 3$, site B, day 2; $N = 1$, site A, day 3), and *Lucilia sericata* ($N = 1$, site B, day 2).

The decay stage started approximately (depending on site) at 72 hours and ended after approximately day 10. Larval masses had spread out from the head and limb-torso junctions and were consuming decaying flesh in an

TABLE 2: Blow fly succession on site B's decaying alligator, Mobile, Ala, USA (August 2002).

Blow fly species	Daily abundance[a,b] (decomposition stage)									
	1	2	3	4	5	6	7	8	9	10
	Fresh	Bloat		Active						
Chrysomya rufifacies	−	−	−	++	++++	+	+	+	+	+++
Cochliomyia macellaria	−	−	+	++	+++	+	+	+	+	−
Chrysomya megacephala	−	+	+	+++	−	+	−	+	−	−
Phormia regina	−	−	−	++	++	+	+	−	−	+
Lucilia coeruleiviridis	+	+	+	+	−	−	+	−	−	−

[a] "−" = 0 adults collected, "+" 1–5, "++" = 6–15, "+++" = 16–25, "++++" > 26.
[b] Day 1 represents the first 24 hours of the study; this 24-hour period began at hour zero (i.e., the time of placement of the carcass in the field at 1000 hours on 5 August 2002) till 1000 hours the next morning on 6 August 2002.

TABLE 3: Blow fly succession on site C's decaying alligator, Mobile, Ala, USA (August 2002).

Blow fly species	Daily abundance[a,b] (decomposition stage)									
	1	2	3	4	5	6	7	8	9	10
	Fresh	Bloat		Active						
Chrysomya rufifacies	−	−	−	−	+++	++	+++	++++	−	−
Cochliomyia macellaria	−	+	−	++	++++	++	++	++	−	−
Chrysomya megacephala	−	−	−	++	++	++	++	++	−	−
Phormia regina	−	−	−	+	+++	+++	++	+	+	−
Lucilia coeruleiviridis	+	++	+++	++	−	−	−	−	−	−

[a] "−" = 0 adults collected, "+" 1–5, "++" = 6–15, "+++" = 16–25, "++++" > 26.
[b] Day 1 represents the first 24 hours of the study; this 24-hour period began at hour zero (i.e., the time of placement of the carcass in the field at 1000 hours on 5 August 2002) till 1000 hours the next morning on 6 August 2002.

anterior-to-posterior fashion. The last part of the alligator to be consumed was the tail; the tongue was never consumed by larvae and eventually dried up. Large numbers of maggots were noted leaving the carcasses at sites A and B on day 5 and a day later for site C (day 6). On day 4, *Chrysomya rufifacies* and *Chrysomya megacephala* visited the carcasses most often. Day 5 was dominated by *Chrysomya rufifacies*, day 6 by *Phormia regina*, and the remaining days by *Chrysomya rufifacies*. *Lucilia coeruleiviridis* rarely visited the carcasses during this stage.

The last stage noted here was the skeletal remains stage. This stage began approximately on day 10 and continued until the bones were collected on 15 September 2002 (day 41). The flesh of the carcasses was largely consumed by the start of this stage. Adult blow flies rarely visited the carcasses during this stage; therefore, are not depicted in Tables 2–4. Dipterous larvae still present were dominated by the black soldier fly *Hermetia illucens* (L.) (Diptera: Stratiomyidae).

Lucilia coeruleiviridis showed a high degree of temporal synchrony among the three sites (Table 4). In contrast, *Chrysomya rufifacies* appeared to be in complete asynchrony among sites with respect to its place in the succession on the alligator carcasses. The remaining three species showed comparison-specific degrees of synchrony/asynchrony among sites. The analysis of variance (Table 5) indicated that only one species, *Cochliomyia macellaria*, showed a significant difference in relative abundance
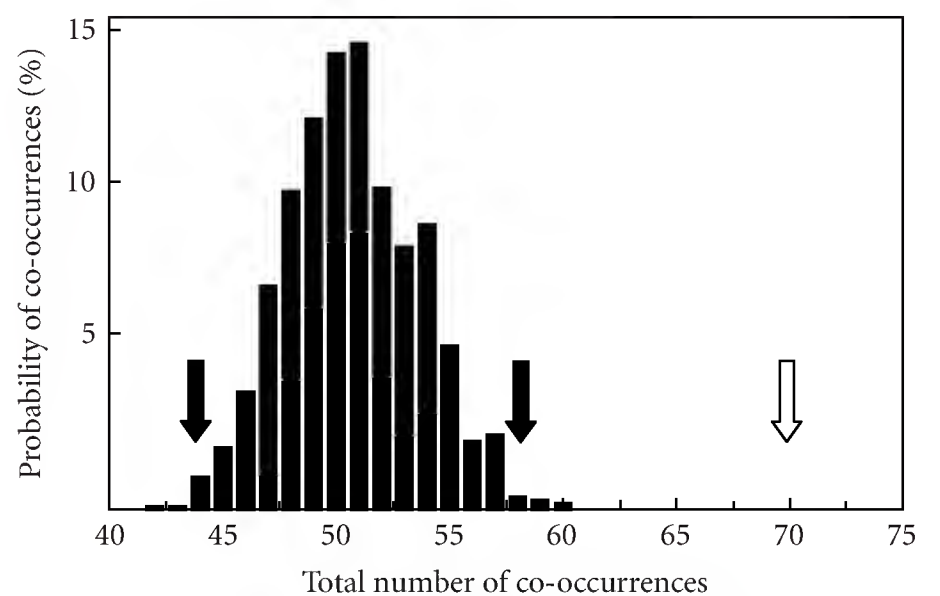
FIGURE 1: Results of 1000 Monte Carlo simulations for blow fly data collected from fly paper at all three alligator-decay sites. The test statistic for this simulation is the total number of co-occurrences (i.e., the number of times a species occurs on the same day in any pair of sites, summed for all species in the analyses). Closed arrows indicate the critical values at the 95.0% level. The observed total number of co-occurrences is shown with the open arrow. Probability of co-occurrence is expressed as a percent.

among sites. This species was collected in greater abundance at site A than sites B and C. The Monte Carlo analysis (Figure 1) showed that combined species co-occurrence

TABLE 4: Dichotomous correlations of blow fly adults over a 10-day period among three sites, each with a single alligator carcass.

Species	Correlation coefficients		
	Site A versus site B	Site A versus site C	Site B versus site C
Chrysomya rufifacies	.50	.272	.534
Cochliomyia macellaria	1*	.356	.356
Chrysomya megacephala	.216	.802*	0
Phormia regina	.6	.816*	.408
Lucilia coeruleiviridis	.816*	1*	.816*

* significant at $P < .05$.

TABLE 5: Analysis of variance for five species of blow fly adults over a 10-day period (block) among three sites (main effect), each with a single alligator carcass.

Species	Mean number of blow flies per day $\pm$ SE[a]			F	P
	Site A	Site B	Site C		
Chrysomya rufifacies	12.3 ± 4.0	10.8 ± 5.5	9.9 ± 4.3	0.12	.889
Cochliomyia macellaria	12.7 ± 3.5a	5.5 ± 2.2b	8.9 ± 4.0ab	4.35	.030
Chrysomya megacephala	8.4 ± 2.1	3.1 ± 1.9	7.0 ± 1.9	2.63	.108
Phormia regina	4.9 ± 2.1	3.0 ± 1.5	6.4 ± 2.8	1.28	.312
Lucilia coeruleiviridis	5.4 ± 2.5	2.6 ± 0.5	8.0 ± 0.5	2.18	.175

[a] For significant ANOVA's ($P < .05$) means with different letters are significantly different at a family error rate of $P = .5$.

occurred at a frequency greater than that expected by a random model. This would indicate at least some synchrony (predictability) of community succession among sites. However, as shown by the correlation analyses, the extent of successional synchrony varied among species and site.

4. Discussion

Watson and Carlton [16, 17] used alligator carcasses as models to study arthropod succession on carrion in Louisiana. Direct comparisons between our findings and of those made in Louisiana are not possible for several reasons. First, our study was done in the summer, and those of Watson and Carlton [16, 17] were done in the spring, fall, and winter. Secondly, the geographical location and vegetation of the sites varied between the studies. Thirdly, the faunal composition of arthropods associated with carrion may be different between the two studies. However, one generalization may be made; *Lucilia coeruleiviridis* is the first blow fly to arrive at alligator carcasses, and even other carcasses in Louisiana and Alabama. Therefore, this blow fly species may be very important in determining the PMI associated with alligators and possibly other carrion. *Lucilia coeruleiviridis* is the first blow fly attracted to decaying dogs in Tennessee [9], white-tailed deer and pigs in Louisiana [17], pigs in Florida [11], and on a variety of mammals in northern Mississippi [15]. However, *Cochliomyia macellaria* is the first to appear on decaying pigs in Texas [31] and in South Carolina [10].

Reasons for the variability of calliphorid succession among our carcasses are unclear, but may be related to subtle differences in the microhabitat in where each carcass was placed or differences in the carcasses themselves. Further attention regarding the floral, chemical, and physical nature of microhabitats needs to be further explored to isolate potential sources of variability in insect succession on carrion. We suspect that the nominal increase in sunlight (lower canopy cover) that site C received may account for the changes in blow fly succession and physical decay observed at this site.

The variability in insect succession on carrion has been attributed to a multitude of variables. For example, carcass size [32], seasonality [9, 18], time since initial exposure of carrion [33], indoors versus outdoors [34], sun versus shade [35], burning [36], burying [37], and hanging [38] have all been investigated. Several studies have evoked the possibility of variation among replicated carcasses, but none of these investigations confirm this suggestion through direct observation (e.g., [6, 13, 39, 40]). Implicit to all carrion studies is the idea that carcasses (of similar physical dimensions) placed in the same habitat at the same time will exhibit limited differences in the rate of decomposition or succession of insects. Our results indicate that this variability needs to be considered in other model carcasses, such as pigs, a model commonly used to establish baseline forensic data [41]. Although our work needs to be repeated at different times of the year and in different habitats, our results suggest that for any particular vertebrate model, replication is critical.

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Research Article

Mate Choice and Copulation Frequency in the Burying Beetle *Nicrophorus quadripunctatus* (Coleoptera: Silphidae): Effect of Male Body Size and Presence of a Rival

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It is widely assumed that there exists a competition between males for mating and that females prefer males with elaborate male traits. Further, such traits are considered to be synonymous with high quality in terms of benefits to females. The number and duration of copulations and the frequency of mate refusal between large and small *Nicrophorus quadripunctatus* males were examined both for single males and for two males competing. The number of copulations was not affected by the size of the male or by the presence of a rival, but there was a significant interaction such that large males increased their number of copulations when a small rival was present. Copulation duration was not affected by male size but was shortened by a rival male. Females rejected copulation attempts of small males more often than of large males, whether the males were alone or paired with a rival. These results suggest that large males have two advantages: they win contests between males and are preferred by females.

1. Introduction

Species which provide biparental care often exhibit monogamous mating behaviors since assistance from the male is essential for successful breeding [1]. Monogamous males fertilize most of the brood, but extrapair fertilizations (EPFs) have been observed in various species [2]. Since EPFs decrease the fitness of male partners, the frequency and/or influence of EPFs is reduced by male partners. Thus, male partners react to EPFs by guarding their mates and frequent copulations [3]. In contrast, females prefer males with some specific traits because fertilization with high-quality males brings about direct and/or indirect benefits [4]. Thus, the success of EPFs depends on both the competition between males and the mate preferences by the female; however, the interaction between these factors is poorly understood.

The complex parental behavior of burying beetles (Silphidae: Nicrophorinae: *Nicrophorus*) has been well studied (reviewed in [5, 6]). *Nicrophorus* exploits small vertebrate carrions as food for its young. Typically, a male-female pair prepares a carcass by burying, removing hair, and rounding it into a ball [7]. Eggs are laid in the soil adjacent to the carrion ball. After hatching, the larvae crawl to the carrion ball, where they are fed by parental regurgitations.

Nicrophorus is generally monogamous [8–11] and displays intense intrasexual competition in both sexes [12, 13]. More than one individual of both sexes often locates a carcass, and usually a single, dominant male-female pair occupies the carcass. Both the males and females defend their carcass and brood even after the larvae hatch by attacking the intruders cooperatively [14–17]. Inferior individuals are occasionally present around the carcass as satellite males [18] and brood parasites [19]. Larger individuals usually win the contest among conspecifics [12, 20]. Because females occasionally reject copulation with males [21], we hypothesize that females prefer to copulate with larger males and that they reject copulation with smaller males. In this study, we examined the number and duration of copulation and the frequency of mate refusal in *N. quadripunctatus*. In addition, we also examined the differences in the frequency of copulations in the presence and absence of other males.
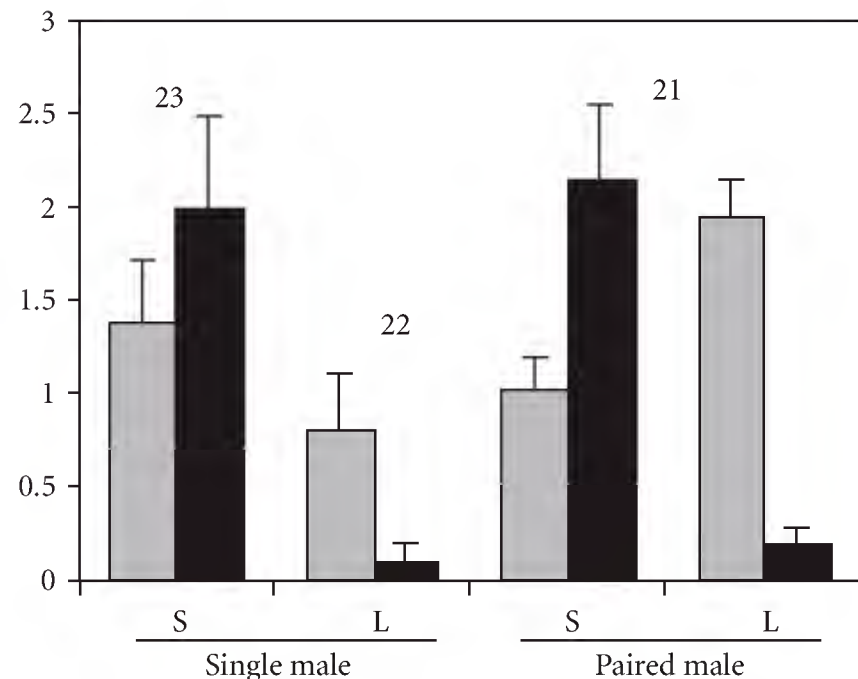
FIGURE 1: Number of copulation attempts (grey bar) and copulation rejections (black bar) for each treatment (mean ± SE). Sample size of the observed copulations is shown at the upper part of each bar.

FIGURE 2: (Mean ± SE) copulation duration for each treatment. Sample size of the observed copulations is shown at the base of each bar.

2. Materials and Methods

All beetles were caught in the field using hanging traps baited with rotten meat. For the experiments, N. quadripunctatus individuals were sorted into large- (pronotal width > 5.5 mm), medium-(5.5 > pronotal width > 4.5 mm), or small- (<4.5 mm) sized classes. The beetles were placed along with a small piece of chicken meat (approx. 15 g) in a plastic arena (50 × 250 × 50 mm). The arenas were maintained under standard laboratory conditions of light and ambient temperatures. The treatments were as follows.

Treatment 1. A large or small male and a medium female were introduced into the arena (large: $N = 22$, small: $N = 23$).

Treatment 2. A medium female and 2 males (a large and a small male) were introduced into the arena ($N = 21$).

The following behavioral interactions between the sexes were recorded for 1 hour. A copulation attempt was recorded when a male attempted to mount the female, and copulation success was recorded when a male mounted the female and successfully inserted his aedeagus. Using a stop-watch, the copulation duration was recorded from the time when the male beetle inserted his aedeagus to the time when he ceased mounting.

3. Results

The frequency of copulation between large and small males was not significantly different ($F = 0.42$, $P = .52$). In contrast, significant interaction between male size and the presence of rivals suggests that large males repeatedly copulated in the presence of small males than that of a single large male ($F = 8.29$, $P < .01$, d.f. = 1, two-way ANOVA, Figure 1). The copulation duration of single males was longer significantly than pair males ($F = 6.57$, $P < .01$, d.f. = 1, ANOVA, Figure 2).
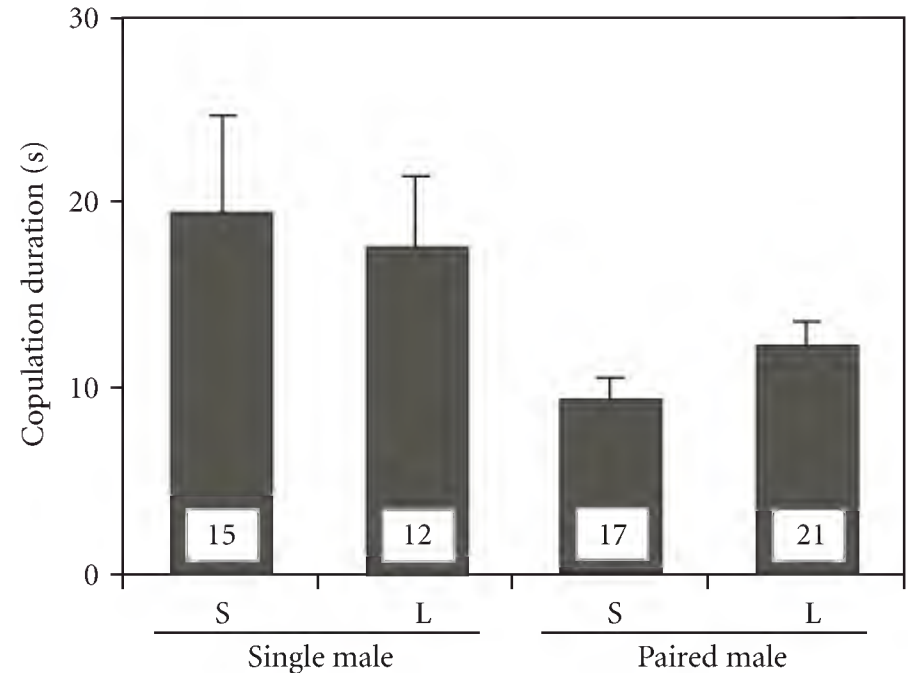
The females displayed two rejection behaviors: holding the abdomen down and moving away from the male [22]. The females tended to accept large males and reject the small males ($F = 36.28$, $P < .01$, d.f. = 1), regardless of the presence of rival males ($F = 0.28$, $P = .59$, d.f. = 1, two-way ANOVA, Figure 1).

4. Discussion

Often two or more male burying beetles are found near a carcass [21]. Thus, intense competition between males has been reported [12, 13, 23]. The dominant male achieves higher paternity, but inferior males often stay around the carcass and copulate in N. vespilloides [18, 19].

Mate rejection is reported in N. vespilloides [22], but the reason for rejection is still unknown. This study revealed that females accepted larger males but rejected smaller males. In burying beetles, conspecific dominant-subordinate relationships are determined by the body size, and larger individuals usually win contests both between males and females [12]. However, even in the presence of only one male, the females prefer larger males over smaller ones as evidenced by the probability of copulation rejection. Thus, the outcome of the contest itself does not affect mate preferences. Since females often equate superior fighting ability with high quality in terms of fitness [24], they are generally attracted to dominant males in many animal species [25], except for a few (e.g., females of Pacific blue-eye fish do not use traits correlated with fighting ability to choose males [26]). The result of N. quadripunctatus coincides with the assumption that females generally prefer dominant males. Females maximize reproductive success by optimizing the "quality" of their mating partners [27], they are assumed to benefit by mating with dominant males.

In male burying beetles, our study revealed that the dominant males copulated more frequently in the presence of inferior males. Territorial (larger) males repeatedly copulated in the presence of other males (Figure 1). Dominant

males of *N. vespilloides* require large number of repeated copulations to achieve sperm replacement for high paternity [18]. The act of repeated copulation was reported only for sperm displacement, and smaller males did not reduce their frequency of copulation in the presence of larger males (Figure 1). The territorial males have the method only with repeated copulations to prevent EPFs in *N. vespilloides* [18, 28]. Territorial males sire a much larger proportion of the brood (60–100%, [18, 19, 29]), and repeated copulations can largely prevent EPFs. In contrast, smaller males are at a disadvantage with regard to fertilization because their possibility of winning contests is low and their rejection as mates is high. Smaller males face a higher possibility of mate rejection, but the number of successful copulations by smaller males is not different from that by larger males without rival males. This demonstrated that smaller males attempted to copulate more frequently than larger males; this also suggested that the smaller males acquired more EPFs by repeated copulation attempts.

It is often stated that males that win contests are the preferred mates because they are of higher quality [27]. It is assumed that females prefer higher quality males for the direct benefits that these males provide, such as paternal care and indirect benefits such as "good gene" [4]. Though large body size has not been confirmed an indicator of good genes in burying beetles, larger individuals usually win the intrasexual contest [12] and acquire higher paternity in burying beetle species (*N. vespilloides*: [29]). There are many conspecific and congeneric intruders even after the larvae have hatched [9], and the parents must guard the brood against them. Larger males usually win contests [12, 13], and pairs can guard their brood more effectively than a single female (*Nicrophorus pustulatus*: [17]). Intruders occasionally take over the owner's brood and kill all the larvae (*Nicrophorus orbicollis*, [30]). Thus, defence against intruders directly affects the reproductive success of the residents. Biparental care improves the protection against intruders provided to the brood by burying beetle parents (*N. pustulatus*: [31]). Trumbo [17] hypothesized that in burying beetles, the threat of infanticide is the primary reason for extended biparental care. In the field condition, sometimes reproductive pairs were determined without intrasexual contest [21]. In such situation, females must select whether they breed with the males presented now or wait for arriving another larger male. Although the relative importance of female choice is still unknown, females will have advantage on selecting larger males for pairs to prevent infanticide effectively. Females can acquire direct benefit by paternal care whether male body size is an indicator of genetic quality or not. In addition, it is said that parental investment is related to certainty of parentage [32], female may insure the paternity of larger male by the rejection of the copulation of smaller males to increase paternal care. Anyway, the effectiveness of mate rejection for females needs further investigation.

This study revealed that females rejected copulation with smaller males, smaller males attempted to copulate more frequently than larger males, and larger males attempted to copulate more frequently in the presence of other *N. quadripunctatus* males. Large-sized males have two advantages: they win contests between males and are preferred by females. Since the adult body size of burying beetles has important effects on reproductive success, the results suggest that the two above-mentioned factors prevent EPFs by smaller males.

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Research Article

Wing Color of Monarch Butterflies (*Danaus plexippus*) in Eastern North America across Life Stages: Migrants Are "Redder" than Breeding and Overwintering Stages

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Monarch butterflies are famous among insects for their unique migration in eastern North America to overwinter sites in Mexico and their bright orange wing color, which has an aposematic function. While capturing migrating monarchs in northeast Georgia, USA, I noticed that many appeared to have unusually deep orange wings. I initiated the current study to compare wing hues (obtained using image analysis of scanned wings) of migrants (captured in 2005 and 2008) to samples of breeding and overwintering monarchs. Consistent with initial observations, migrants had significantly lower orange hues (reflecting deeper, redder orange colors) than breeding and overwintering monarchs. There was also a difference in hue between sexes and a relationship with wing size, such that larger monarchs had deeper, redder hues. The reasons for the color difference of migrants are not apparent, but one possibility is that the longer-lived migrant generation has denser scalation to allow for scale loss over their lifespan. Alternatively, this effect could be confined to the subpopulation of monarchs in the Southeastern United States, which may not be well represented at the Mexican overwintering sites. In any case, this discovery highlights the many questions emerging on the significance of wing color variation in this species.

1. Introduction

The monarch butterfly (*Danaus plexippus*) is one of the world's most well-known insects, being easily identifiable by its bright orange and black wing colors (Figure 1). It is the posterchild for insect conservation, and its image even graces the front cover of this journal. One of the reasons for this attention is certainly the amazing annual migration of the population in eastern North America, which starts in breeding grounds in Canada and the northern United States, and ends some 3000 km away at a select few overwintering sites in the mountains of Central Mexico [1, 2]. There, they form massive clusters of millions of butterflies, and wait until spring, when they remigrate northward to repopulate the breeding grounds [3, 4]. This unique life cycle is completed in many generations. Breeding monarchs normally live for one month, undergoing several generations per summer. The final generation of the late summer is the one that undertakes the migration and overwintering phase, so that these individuals live up to 9 months [5]. Over many decades, a considerable body of literature has been developed around this unique insect, although recent discoveries are revealing how many questions about it yet exist. For example, the functional significance of the orange coloration of the monarch's wings has long been known to be a warning to predators of its toxicity from the cardenolides the larvae sequester from the host plants, which are members of the genus *Asclepias* [6]. However, recent work using modern image analysis techniques has demonstrated there is considerable variation in the degree or shade of orange color among monarchs [7]. This discovery together with the use of today's image analysis computer programs that can easily quantify color variation on images of butterfly wings, opens up a new body of questions regarding the significance of this variation.

The current study was initiated because of certain casual observations regarding monarch butterfly wing color. Specifically, I noticed that monarchs captured during the fall migration sometimes appeared (with the naked eye) to have
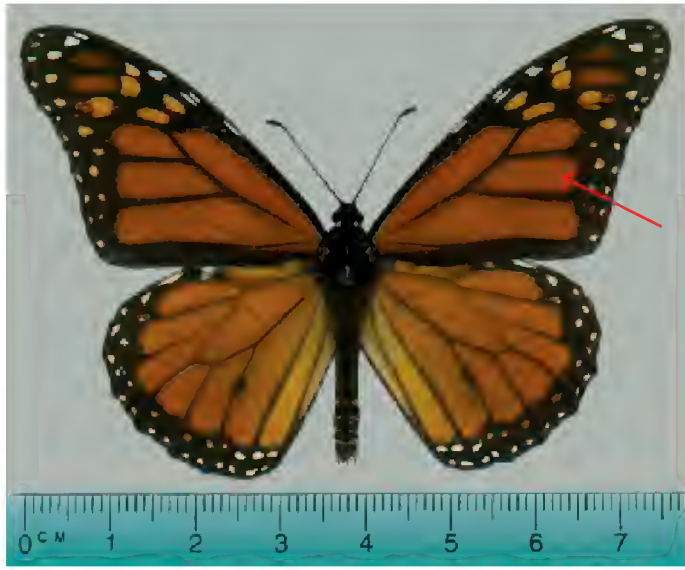
FIGURE 1: Monarch butterfly, *D. plexippus*. This individual, a male, was captured in September 2005 by AKD. Arrow indicates the cell used for measuring color in this study.

more reddish-colored wings than those reared in lab experiments [7, 8] and than those that had been captured during the summer months. I subsequently developed the current project aimed at determining if this difference was real, by using an image analysis approach to objectively quantify the orange color of monarch wings, similar to that performed recently [7]. With these data, I statistically compared wing colors of monarchs captured during migration to a sample of breeding monarchs and to a collection of overwintering monarchs, and I report the results of this exploration here.

2. Methods

2.1. Butterfly Sources. Three sets of monarch butterfly specimens were examined in this study. The first group (n = 39) was collected during the summer of 1997 in Minnesota and Wisconsin (hereafter called "breeding" group), between July 11 and August 30, although 85% of these were collected before August 15. These specimens had been stored at $-20°$C (individually in glassine envelopes) since capture. The second set were 75 individuals collected at two of the Mexican overwintering sites in February 2008 (hereafter called "overwintering" group). These were netted from random clusters and brought to the lab (with all applicable permits), where they were also stored at $-20°$C. The third group consisted of monarchs captured during the fall migration in 2005 and 2008 (n = 29 and 31; hereafter called "migrant" group) and were killed via freezing immediately after capture and stored at $-20°$C. All migrants were captured while nectaring on blooming vegetation near Athens, Ga, USA ($33.9°$N–$83.4°$W), during the periods of October 14–22, 2005 and September 11–29, 2008.

2.2. Scanning and Processing Wings. All frozen stored specimens were thawed and their forewings were removed for scanning. Scanning procedure generally followed procedures established in prior work [7, 8]. Wings were placed face down on a standard flatbed scanner connected to a laptop computer, and scanned at 300 dpi. The exposure settings on the scanner were set so that the original wing color

was maintained (i.e., so that the scanner software did not manipulate the images). All forewings were scanned in this manner (i.e., with this scanner and with these settings). When scanned, the sex of each monarch was also recorded.

When all wings were scanned, each forewing image was imported into the image analysis software, FoveaPro (Reindeer Graphics, Inc., NC, USA) (http://www.reindeergraphics.com/), which works in the Photoshop environment. This program is ideally suited to measure color, and has been used by the author in prior studies [7–11]. Wing color measurement generally followed procedures established in prior work that focused on this species [7] with slight modification. Briefly, the central wing cell of the right forewing (Figure 1) was selected using the "magic wand tool." I considered this selected area to be representative of the orange color of the entire wing. Further, choosing this location to measure provided a convenient border for the selected area. However, since female monarchs have more black scales over their wing veins, which makes their veins appear thicker than males, this means that in general, the selected area was smaller in females than in males. Next, a color-measure routine was initiated which returned the average hue, saturation, and brightness values for all pixels selected (usually between 5 and 10 thousand pixels). In this case, only the hue values were retained, which is in contrast to prior work, where the saturation score was the focus [7]. The hue was more appropriate to examine in this study since the magnitude of the color variation was thought to be large based on initial observations of migrant monarchs (i.e., ranging from pale yellow to orange to dull red). Hue is measured in degrees (i.e., 0–360), with 0 representing perfect red, and in this study the scores tended to be between 25 and 40 (see results). Finally, a secondary routine was run which returned the area of the entire forewing in mm^2, based on prior calibration of the software using a scanned ruler.

2.3. Data Analysis. Since two sets of migrants were obtained in this study, I initially examined these data for possible differences using a two-sample t-test, where the wing hue was the dependent variable and the year was the independent. This test revealed no significant variation (df = 58, t = -0.811, P = .421), therefore these data were pooled for subsequent analyses. I then used general linear modeling procedures to examine the possible variation in monarch wing color (hue) among life stages, that is, breeding, migrating, and overwintering. Thus, the analysis included wing hue as the response variable, "group" as a categorical independent, as well as gender. Finally, butterfly size (indexed by forewing area) was included as a continuous covariate. All two-way interaction terms were initially included but removed if found nonsignificant. All analyses were conducted using Statistica 6.1 software [12].

3. Results

Across all monarchs examined in this study (n = 174), there was considerable variation in wing hue scores, which ranged from 25.3 to 40.1 degrees (Figure 2). Lower hue scores

Table 1: Summary of general linear model examining factors influencing wing hue of monarch butterflies in this study. Groups were "breeding," "overwintering," and "migrating." Nonsignificant interaction terms (i.e., where $P > .05$) were removed.

Variable	df	MS	F	P-value
Sex	1	475.93	187.47	<.0001
Group	2	391.56	154.23	<.0001
Wing area	1	12.35	4.87	.0288
Sex*group	2	10.99	4.33	.0147
Error	167	2.54	—	—
Total	173	—	—	—

Figure 3: Average wing hue score (in degrees) across all groups for male ($n = 107$) and female ($n = 67$) monarchs in this study. Error bars represent 95% CI.
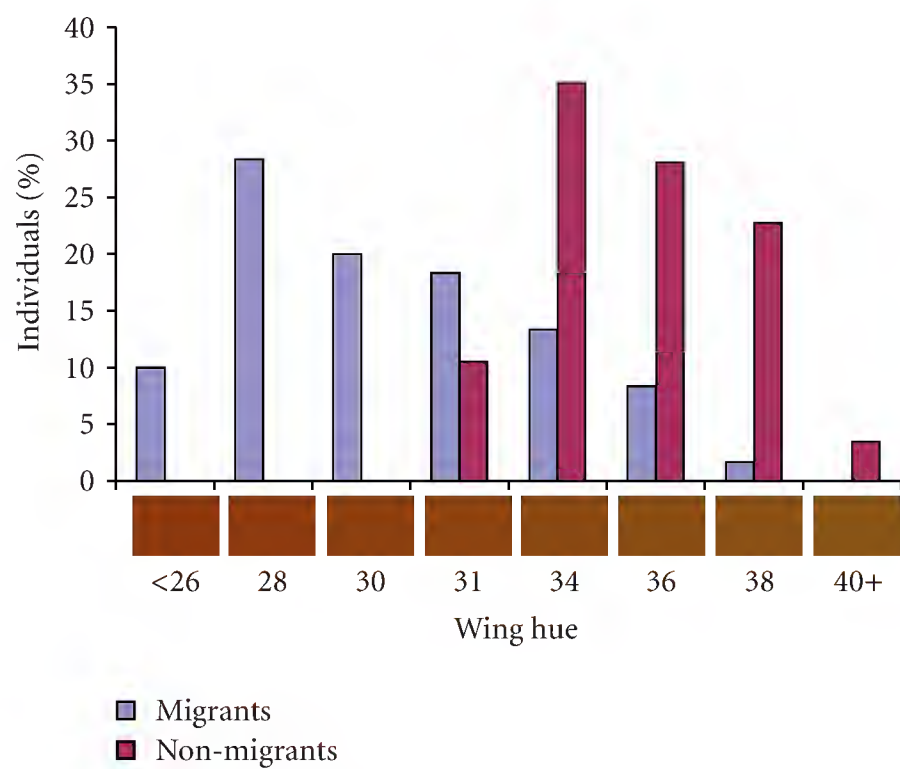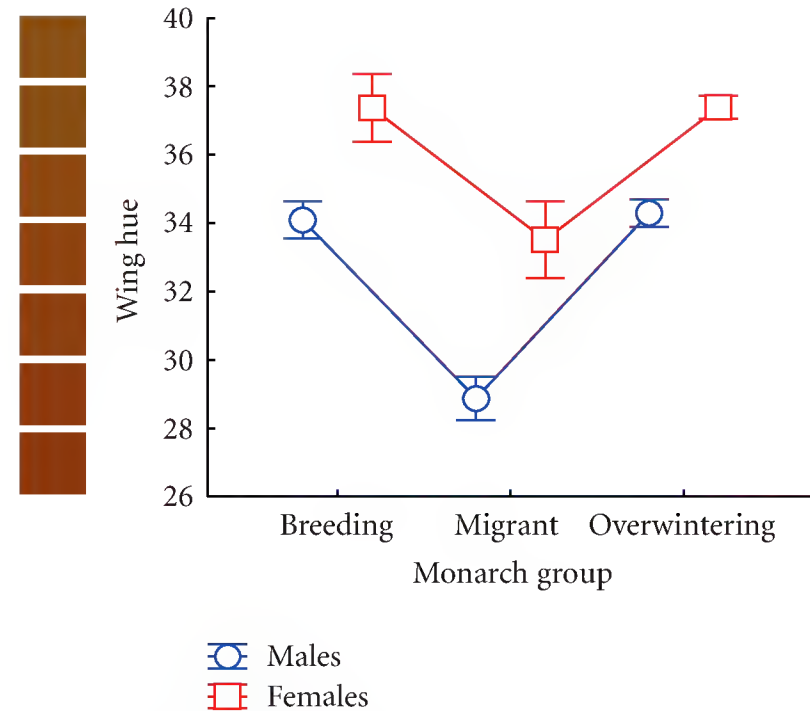

Figure 2: Frequency distribution of wing color (hue score, measured in degrees) observed among all migrating ($n = 60$) and nonmigrating ($n = 114$) monarchs. Colored scale included which shows the hue scores of each category.

differed among groups, as evidenced by the significant interaction term of sex*group in the final model ($P = .0147$; Table 1). This interaction effect can also be seen in Figure 3, where the overall difference between male and female hue scores was the greatest in the migrant group (4.6 degrees difference), versus 3.3 degrees for breeding and 3.1 degrees for overwintering monarchs.

Finally, there was a surprising effect of wing size on hue score ($P = .0288$; Table 1). This effect was negative, such that large wings tended to have lower hue scores. Put another way, larger monarchs tended to be more red-colored. The interaction terms of wing area*sex and wing area*group were not significant, meaning that this relationship held for both sexes and across all life stages.

4. Discussion

The results of this exploratory study corroborate the initial observations made of the wings of migrating monarchs. That is, migrating monarch butterflies appear to have significantly deeper orange wings than they do during the summer. Further, I observed this phenomenon in two separate migrations (2005 and 2008), and in both cases the migrants' wing colors were similarly deep orange or nearly red (and were statistically similar). Thus, this phenomenon is not random but appears real. This discovery then represents the first morphological trait shown to be different among the final summer migratory generation and the mid-summer breeding generations. The reason for this difference is not clear, although one could speculate that the deeper orange color may help to absorb solar energy, which would enable flight at lower temperatures. Alternatively, the deep orange could reflect some physiological shift by larvae of the final generation (i.e., allocating resources from other processes to pigment production).

Besides the differences in wing color found between migrating and breeding monarchs, a more surprising result

reflect deeper orange colors, almost nearing red, while the higher scores indicate generally paler, more yellowed wings. In the statistical analysis of wing hue, there was a significant effect of group ($P < .0001$; Table 1) in that migrants had significantly lower hues than all other life stages (Tukey's pos-hoc test; Figure 3). The mean hue of migrants was 30.5 and the hues of breeding and overwintering monarchs were 35.2 and 35.6, respectively. To visually depict this large difference between migrants and other groups, Figure 4 shows selected forewings from each group. Note the color of the orange wing cells in each group. Furthermore, the distribution of migrant monarchs is graphed separately from other nonmigrant groups in Figure 2 to highlight the large difference in wing color of migrants.

In addition to the differences among stages, I also discovered that males differ in wing hue from females ($P < .0001$; Table 1). In all three life stages, males had significantly lower hue scores than females (Figure 3). In other words, the orange color on male monarch wings tends to be deeper, or more reddish, while female monarchs have a paler orange color. However, the magnitude of this gender difference
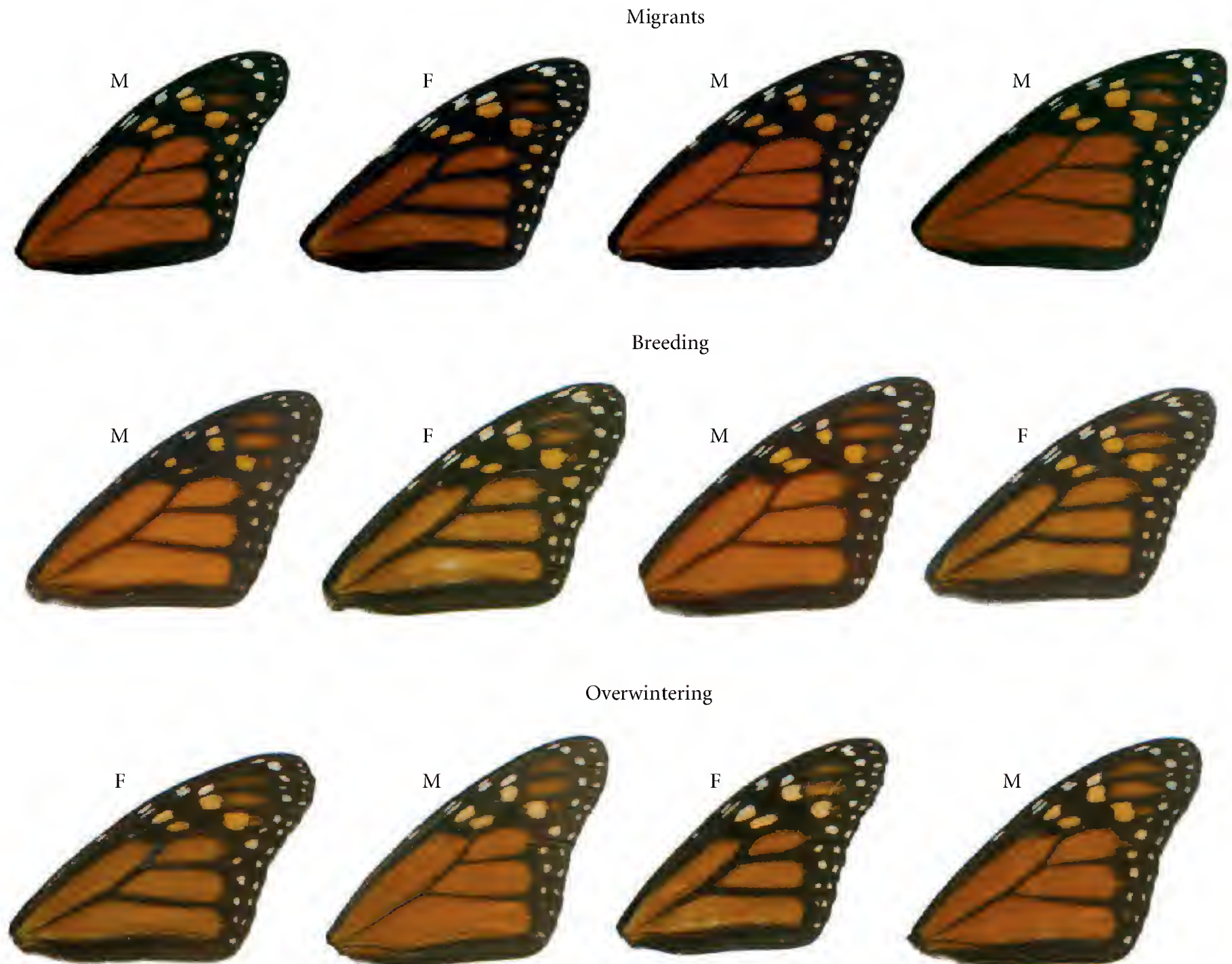
FIGURE 4: Forewings of selected migrant, breeding, and overwintering monarch butterflies. Note the difference in orange color between migrants and all others. All wings were scanned with the same scanner using the same settings. Males (M) and females (F) are indicated.

of this study was that the wing color of *migrating* monarchs did not resemble that of *overwintering* monarchs. The overwintering monarchs all appeared to have the same pale-orange color of breeding monarchs. How then, is this possible if they are all cohorts of the same generation? Does the migrant wing color fade over time while at the overwintering locales? While there is little research on this subject, the answer to this question would probably be yes. This generation is certainly the longest lived by far, and it may make sense that the migratory generation produces heavily pigmented wings to account for the eventual scale loss over time.

A second explanation may be that this phenomenon (of deeper-orange migrants) is unique to the population of monarchs in the southeastern United States, and that monarchs from this area are not well represented at the Mexican overwintering site. In support of this idea, recent work examining migration routes of monarchs indicated that monarchs migrating along the eastern seaboard have a reduced chance of reaching the overwintering sites than those in the interior United States [13]. Furthermore, migrating monarchs captured and tagged in the Florida Panhandle

have only a 1 in 4000 chance of being recovered at Mexico overwintering sites (cited in [13]). In contrast, the normal Mexican recovery rate for tagged monarchs from the eastern population as a whole is 1 in 250 [14]. Thus, monarchs migrating through Georgia may not be well represented at the Mexican overwintering site. Clearly, future work should examine wing colors of migrating monarchs from other regions to determine how widespread this phenomenon is. Only then, will we know if this phenomenon is unique to the southeast, or if all migrants display this trait. If the latter is true, then the discrepancy between migrant and overwintering wing color would become even more perplexing.

There were two other surprising findings in this study. First, I found that males and females differ statistically in wing hue, with males tending to have a deeper orange color than females. This species was already known for another sexually dimorphic trait, in that females have a greater degree of black pigmentation [8], see Figure 4, but it would seem that the sexes differ in other features as well. Secondly, there was an unexpected relationship found (although wing size was included as a covariate in the analysis to account

for this possibility) between wing size and hue, such that larger individuals tended to have a deeper orange to dull-red color. Like most butterflies, large body size in monarchs is generally considered an indicator of fitness [15, 16], so deeper orange wing color may be a sign of more robust individuals. However, a greater understanding is needed of the causal mechanisms behind wing color formation before this idea can be fleshed out further.

Clearly, there are a number of findings from this work that open up a large number of additional questions on the significance of monarch butterfly wing color, and which will take much more study to address. However, since even these simple comparisons of wings between breeding, migrating, and overwintering monarchs here proved fruitful, the possibility for further exciting discoveries in this research avenue is high. This is especially true since the discoveries made here only serve to emphasize the large gaps that remain in our knowledge of the biology of this unique and charismatic insect.

Acknowledgments

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Research Article

Comparative Study of the Morphology of the Ovipositor of *Platygaster diplosisae* (Hymenoptera: Platygasteridae) and *Aprostocetus procerae* (Hymenoptera: Eulophidae) Two Parasitoids Associated with the African Rice Gall Midge, *Orseolia oryzivora* (Diptera: Cecidomyiidae)

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We studied the morphology of the ovipositor of *Platygaster diplosisae* (Hymenoptera: Platygasteridae) and *Aprostocetus procerae* (= *Tetrastichus pachydiplosisae*) (Hymenoptera: Eulophidae), two parasitoids associated with the African rice gall midge (AFRGM), and *Orseolia oryzivora* (Diptera: Cecidomyiidae). Scanning electron microscope techniques were used for this study. The ovipositor of *P. diplosisae* was short (40 µm), and most of the sensillae found on it were mechanoreceptors and located on the distal portion of the 3rd valvulae. These sensillae may be involved in selection of an egg or larval host. The shortness of this ovipositor may be an adaptation to a host whose egg envelope thickness is not more than 0.7 µm. The ovipositor of *A. procerae* was 30 times (1.2 mm) the length of the *P. diplosisae* ovipositor. It was not only well equipped with mechanoreceptive sensillae, but these sensillae were very diverse and distributed along the length of the valvulae. The 10 denticulations of the lancet of this ovipositor allow this parasitoid to exploit hosts that are not otherwise readily accesible. These two parasitoids share the same resource by infesting different life stages of the host. The ovipositor of each species of parasitoid enhanced resource sharing, due to its length and its sensillae type and distribution.

1. Introduction

The African rice gall midge (AfRGM), *Orseolia oryzivora* Harris & Gagné (Diptera: Cecidomyiidae), is an insect pest indigenous to Africa. It was considered a minor pest prior to the 1970s but has since caused increasingly severe damage to rice crops [1].

The young larva feeds on tillers at the growing point of the rice plant and induces the plant to form an oval, hollow gall. Each gall prevents production of a panicle. The amount of yield loss caused by the gall midge larva varies among rice varieties. Nacro et al. [2], and Williams et al. [3] showed that an increase in 1% in the percentage of tillers with galls at the stem-elongation stage reduced yield by 2 to 3%.

It has been reported that early and synchronized plantings of rice reduce the damage by the AfRGM [1]. Unfortunately, these cultural control methods are very often insufficient because of problems with water management and the conflicting management of both upland cereal crops and irrigated rice. The use of insecticides to control AfRGM is not ideal because of the cost, the risk to human health and the environment, and the destruction of natural enemies [4]. Furthermore, only systemic insecticides are likely to be effective in the control of the midge because of its feeding
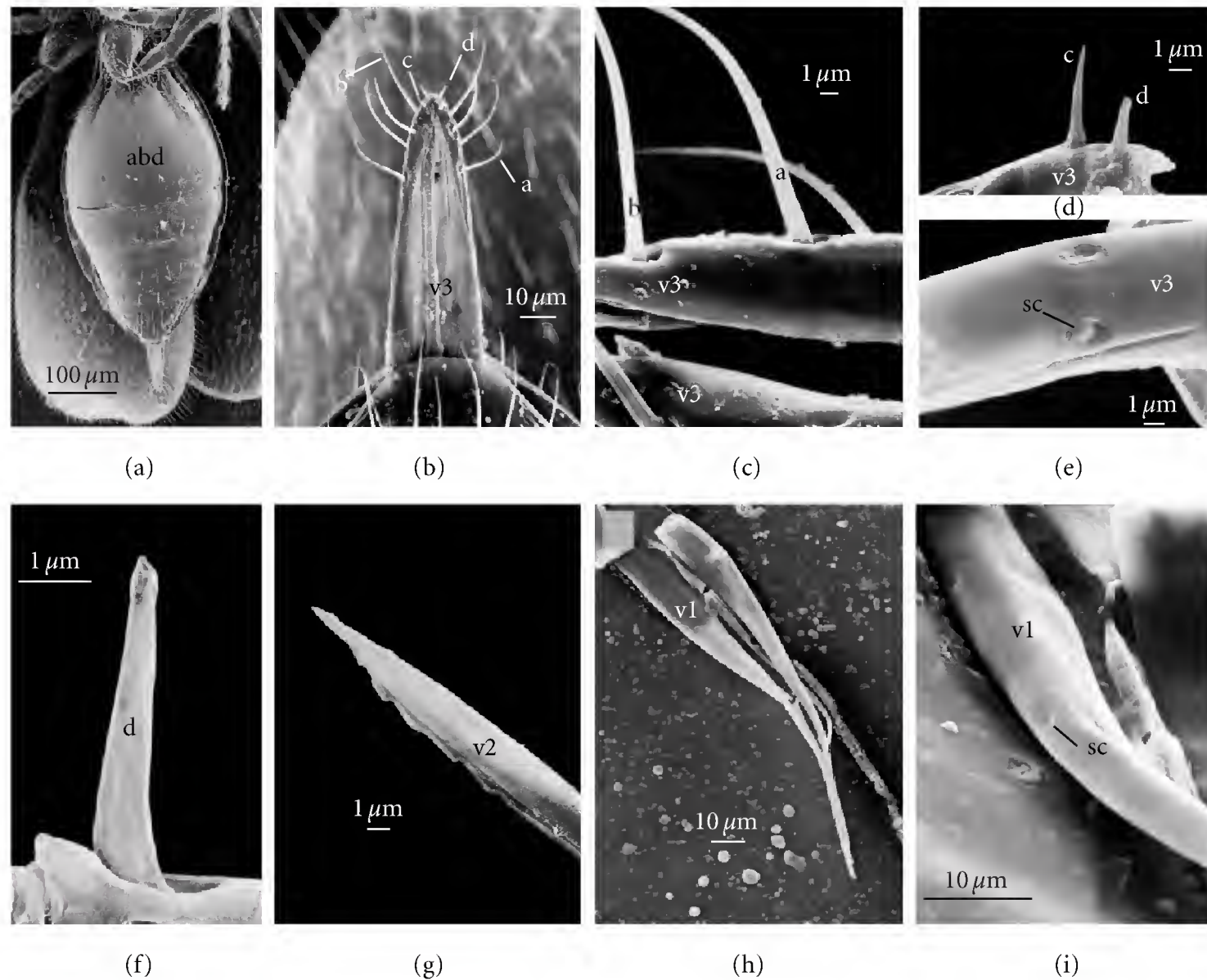
FIGURE 1: Ovipositor of *P. diplosisae*. (a) View of the abdomen showing the ovipositor at its distal end. abd: abdomen. (b) Front view of the ovipositor of *P. diplosisae*. Several sensillae are seen on the 3rd valvulae. a: trichoid sensillum of type a; b: trichoid sensillum of type b; c: sensillum of type c; d: sensillum of type d. (c) Valves 3 showing 2 types of trichoid sensillae. a: sensillum of type a; b: sensillum of type b. (d) Distal end of the 3rd valvulae with 2 types of trichoid sensillae. c: sensillae of type c; d: sensillae of type d. (e) Two campaniform sensillae located at the proximal portion of the 3rd valvulae. v3: 3rd valvula; sc: campaniform sensillum. (f) Sensillum of type d observed on the distal end of the 3rd valvula. d: sensillum of type d. (g) Distal end of the ventral part of a 2nd valvula viewed in profile. Five denticles are seen on this valvula. v2: 2nd valvula. (h) 1st valvula of the ovipositor. They appear large in their proximal portion and more and more slender near their distal end. v1: 1st valvula. (i) Two campaniform sensillae on the proximal portion of a 1st valvula. v1: 1st valvula.

habit inside plant tissue. The conservation of natural enemies of the AfRGM may be a good alternative to insecticidal control.

So far, little is known about the predators of AfRGM. Some egg predators have been reported [1]. These include tiny predatory mites (*Neoseiulus* sp., Phytoseiidae), the bug *Cyrtorhinus viridis* Linnavuori (Miridae), and the sword-tailed crickets *Anexipha longipennis* Serville, and *Trigonidium cicindeloides* Rambur (Gryllidae). Ladybird beetles (Coccinellidae) and the long-horned grasshopper *Conocephalus* (Tettigoniidae) are also egg predators. Two common parasitoids are known to be associated with the AfRGM. These are *Platygaster diplosisae* Risbec (Hymenoptera: Platygastridae) and *Aprostocetus procerae* Risbec (=*Tetrastichus pachydiplosisae*) (Hymenoptera: Eulophidae). These two parasitoids are the primary biological control agents. *P. diplosisae* is a gregarious larval parasitoid whereas *A. procerae* is a solitary pupal parasitoid of the AfRGM [5]. *P. diplosisae* oviposits inside the eggs or the larvae of AfRGM. The parasitoid's larvae hatch inside the young AfRGM larva. They feed inside the larva and kill it when it is fully grown. They then pupate inside the corpse, from which the adults emerge. The adults

cut one or more very small exit holes in the gall and disperse. The adult *A. procerae* lays its eggs onto AfRGM pupae, or occasionally onto large larvae. It does this by piercing through the wall of the gall with the tip of its abdomen. The host is stung and paralyzed by the female parasitoid as the egg is laid. *A. procerae* feeds on, rather than inside, the host, and only one larva develops on each host. After it has finished feeding, the parasitoid larva changes into a pupa inside the gall. The adult that emerges cuts an exit hole in the gall to escape. Cumulative parasitism due to these two hymenopterans has been reported to reach 77% [6–9]. However, sometimes, such a high level of parasitism occurs too late to prevent damage by the pest.

The ovipositor of parasitic hymenopterans is primarily used to deposit an egg into or onto a host [9]. The structure of the ovipositor in different species of parasitic hymenoptera varies both in length and in its arrangement on the terminal metasomal segments. The general organization of the ovipositor includes 3-paired valvulae (1, 2, and 3), one paired valvifers. The paired 1st valvulae and the 2nd paired valvulae are fused in their distal portion to form the lancet, which is the piercing organ.

This study compares the ovipositor of *A. procerae* and *P. diplosisae* in terms of morphology and function of the associated sensillae. Furthermore, we hypothesize about the possible effect of the sensillae richness and diversity on the parasitism rate of *A. procerae* and *P. diplosisae*.

2. Material and Methods

Adult parasitoids were captured from irrigated rice fields in Burkina Faso and kept in a 90% alcohol solution and sent to France where all the laboratory work was completed. The average age of the specimen was 7. We used about 100 individuals of each parasitoid species in 5 replicates. Unfortunately, due to the smallness of the ovipositor of *P. diplosisae* only a few samples were observed under electron microscope. Ovipositors of *A. procerae* and *P. diplosisae* were dehydrated in successive alcohol solutions (70%, 80%, 95%, and 100%) and acetone solutions (50%, 70%, 90%, and 100%). Ovipositors were then mounted on a lead object-holder. Samples were critical point dried in a Balzers CPD 010 apparatus with liquid CO_2 gas and then gold palladium coated with a JEOL JFC-100 sputter. These samples were observed under a JSM 6400 electron-scanning microscope (JEOL Ltd, Japan).

3. Results

3.1. Description of the Ovipositor of Platygaster diplosisae. The ovipositor of *P. diplosisae* measures $40\,\mu$m in length (Figure 1(a)).

3.1.1. 3rd Valvulae. The paired 3rd valvulae are $40\,\mu$m in length and protect the 1st and 2nd valvulae when the ovipositor is at rest (Figure 1(a); see arrow). 3rd valvulae are connected distally, which causes a cone that has a slightly flat peak (Figure 1(b)).

3.1.2. 1st and 2nd Valvulae. The two pairs of 1st and 2nd valvulae are fused and form the ovipositor stylet (Figure 1(g)).

At rest, this ovipositor stylet is entirely embedded in the cavity of the 3rd valvulae (Figure 1(b)). The paired valvulae are larger in their proximal portion and come to a sharp point distally (Figure 1(h)). The extremity of the paired 2nd valvulae, also called the lancet, is equipped with five denticles (Figure 1(g); see arrow).

3.2. Sensillae on the Ovipositor of P. diplosisae. The sensillae on the ovipositor are relatively simple.

3.2.1. 3rd Valvulae. Two-thirds of these sensillae are campaniform sensillae whose external process is a dome embedded in a cuticular depression (Figure 1(e)). The distal 1/3 of the 3rd valvulae possesses four types of sensillae (Figures 1(b), 1(f)). Following is a list and description of the four sensillae types:

(i) 3 trichoid sensillae of type a, nonaligned. They are slightly curved and measure $12\,\mu$m in length.

(ii) 1 trichoid sensillum of type b, slightly straighter than the other three but as long as them ($13\,\mu$m length) (Figures 1(b), 1(c)).

(iii) 1 unique sensillum of type c, $3\,\mu$m in length, so 4 times shorter than the trichoid sensillae described early (Figures 1(b), 1(d)). Unlike the trichoid sensillae, the base of the type c sensillum is not embedded in a cuticular depression. Its diameter is more continuous and its extremity is less sharp.

(iv) 1 unique sensillum of type d, located near the sensillum of the type c (Figures 1(b), 1(d), 1(f)). It measures $2.5\,\mu$m. The base is embedded in a depression similar to the trichoid sensillum. It is grooved and its extremity ends with a bludgeon. It measures $2.5\,\mu$m in length. This type of sensillum resembles the chaetica of type 1 observed by Van Baaren [10], on the antenna of *Epidinocarsis lopezi* (de Santis) (Hymenoptera: Encyrtidae) a solitary endoparasitoid of the cassava mealybug, *Phenacoccus manihoti* Matile-Ferrero (Homoptera: Pseudococcidae). The extremity of this sensillum which was not explored by scanning electron microscopy could bear a pore. The trichoid sensillae, the campaniform sensillae and the sensillum of type c may be mechanoreceptors.

3.2.2. 1st and 2nd Valvulae. The 1st paired valvulae are equipped with campaniform sensillae aligned on a line that runs the length of the valvulae (Figure 1(i)). These sensillae are the same type as those observed on the distal two thirds of the 3rd valvulae. These are mechanoreceptive sensillae similar to those described on the ovipositor valvulae of several hymenopteran parasitoids [11].

The size and the low number of ovipositors examined did not allow us to determine whether the 2nd valvulae are equipped with sensillae.

Table 1 summarizes the different types of sensillae and their possible function.

3.3. Description of the Ovipositor of A. procerae. The ovipositor of *A. procerae* consists of a stylet surrounded by the paired 3rd valvulae (Figure 2(a)).

3.3.1. 3rd Valvulae. These valvulae are largest proximally, but slightly sharp distally (Figure 2(b)). The internal surface of the 3rd valvulae has many cuticular spines (Figure 2(d); see arrow).

3.3.2. 1st and 2nd Valvulae. The ovipositor stylet of *A. procerae* is 1.2 mm in length, surrounded by the paired 3rd valvulae. The paired 2nd valvulae are coupled by the 1st valvulae. A sliding system allows the lancet formed by the fusion of the paired 2nd valvulae to move backward and forward. The lancet bears a notch that limits the movements of the paired 2nd and 1st valvulae. This lancet is $92\,\mu$m long and bears 10 denticles on its external surface (Figures 2(e), 2(f)). These denticles are increasingly smaller from proximal to distal, which gives the perforating system of the ovipositor its sharp form.
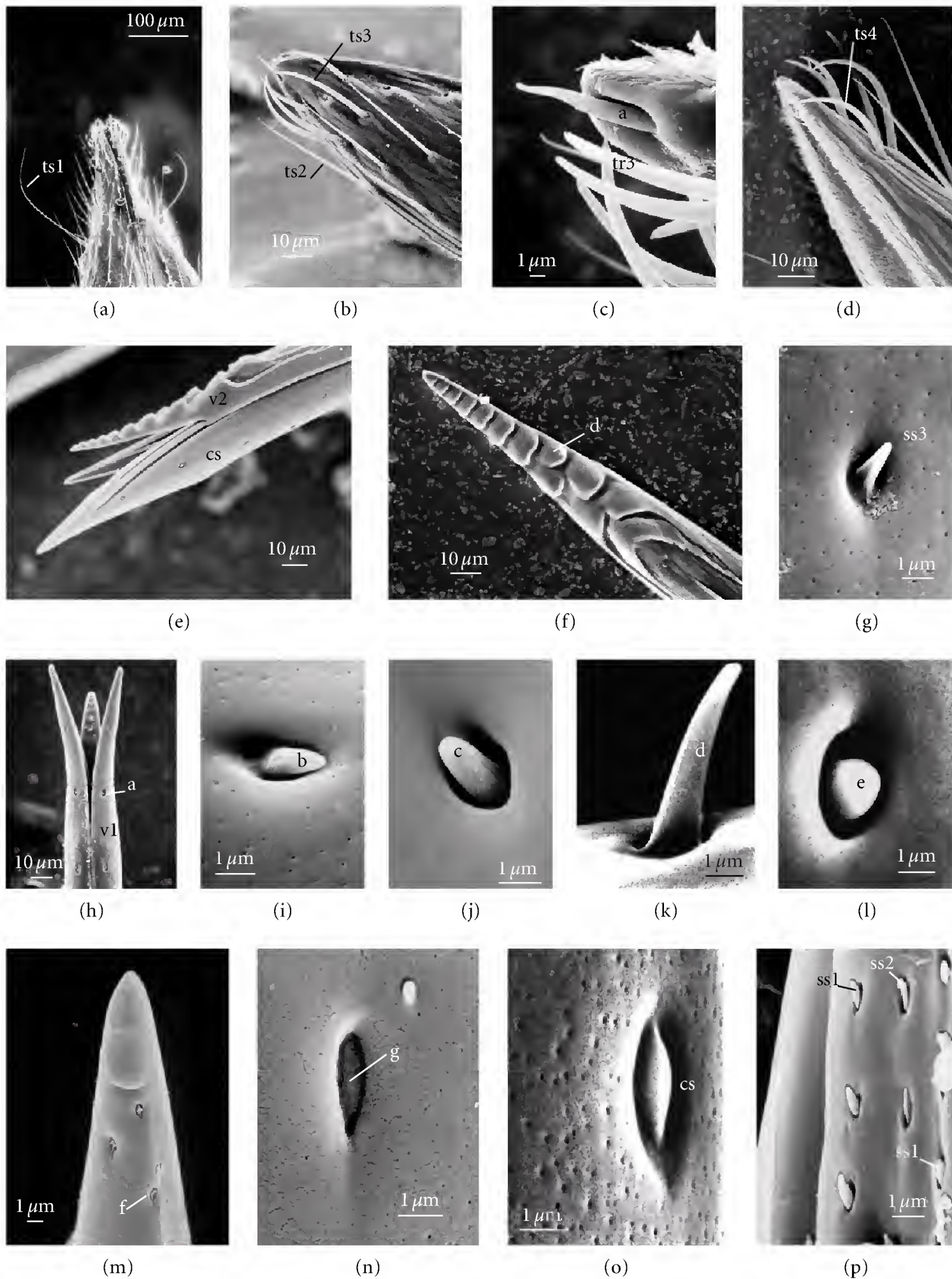
FIGURE 2: Ovipositor of *A. procerae*. (a) Extremity of the abdomen of *A. procerae* showing the valvulae at the distal end of the stylet. ts1: trichoid sensillum of type 1. (b) External surface of the distal end of a 3rd valvula bearing several types of trichoid sensillae; ts2: trichoid sensillum of type 2; ts3: trichoid sensillum of type 3. (c) Extremity of a 3rd valvula showing 3 sensillae of type 3. a: sensillum of type a. (d) Internal surface of the distal end of a 3rd valvula, viewed in profile. The interior of the valvula is empty. A trichoid sensillum of type 4 is seen. ts4: trichoid sensillum of type 4. (e) Distal extremity of the stylet of the ovipositor viewed in profile. The 2nd valvulae are carried by the 1st valvulae. v2: 2nd valvula; cs: campaniform sensillum. (f) Distal end of the dorsal surface of a 2nd valvula showing 10 denticulations making a united lancet. d: denticulation. (g) Styloconic sensillum of type 3 on the external surface of the proximal part of a valve 2. ss3: styloconic sensillum of type 3. (h) Distal extremity of the ventral part of the stylet of the ovipositor viewed in front. v1: valve 1; a: campaniform sensillum of type a. (i) Sensillum of type b located on the half proximal part of the external surface of a valve 1. b: sensillum of type b. (j) Sensillum of type c located on the half proximal part of the external surface of a valve 1. c: sensillum of type c. (k) Sensillum of type d, observed on the external surface of the proximal part of a valve 1. d: sensillum of type d. (l) Sensillum of type e, located at $120\,\mu$m of the distal extremity of a valve 1. e: sensillum of type e. (m) External surface of the distal extremity of a valve 1. The three sensillae of type f are distributed in triangle. f: sensillum of type f. (n) Sensillum of type g located at the half proximal part on the external surface of a valve 1. g: sensillum of type g. (o) Campaniform sensillum located at the 2/3 proximal of the a valve 1. cs: campaniform sensillum. (p) Three rows of styloconic sensillae of type 1 and 2 on the external surface of the distal extremity of a valve 3. ss1: styloconic sensillum of type 1; ss2: styloconic sensillum of type 2.

TABLE 1: Distribution of the sensillae on the ovipositor of *P. diplosisae*.

Type	Localization	Number	Length(μm)	Existence of an apical pore	Possible function
Campaniform mechanoreceptors	2/3 proximal of 3rd valvulae	—	—	—	—
Mechanoreceptors	Entire length of 1st valvulae 3rd valvulae	—	—	—	—
Trichoid a	1/3 distal	3	12	No	Mechanoreceptors
Trichoid b	Distal extremity of 3rd valvulae	1	13	No	Mechanoreceptors
c	Distal extremity of 3rd valvulae	1	3	No	Mechanoreceptors
d	Distal extremity of 3rd valvulae	1	2.5	Not sure	Chemoreceptors

TABLE 2: Distribution of the sensillae on the ovipositor of *A. procerae*.

Type	Localization (μm)	Number	Extremity	Length	Existence of an apical pore	Probable function
Trichoid 1	Proximal portion of 3rd valvula	1	Sharp	104	No	Mechanoreceptor
Trichoid 2	58 μm from distal extremity of 3rd valvula	The most common of the trichoid type	Slightly sharp	51	No	Mechanoreceptor
Trichoid 3	42 μm from the distal extremity of 3rd valvula	—	Slightly rounded	38	No	Mechanoreceptor
Type a	Distal extremity of 3rd valvula	1	Rounded	5.3	No	Mechanoreceptor
Type b	Distal of 1st valvula	Few	Rounded	0.9	No	Mechanoreceptor
Type c	Half proximal of 1st valvula	Few	Rounded	0.8	No	Mechanoreceptor
Type d	Proximal portion of 1st valvula	1	Rounded	0.47	No	Mechanoreceptor
Type e	120 μm from the distal extrimity of 1st valvula					
Type f	5 μm from the distal extremity of 1st valvula 3	3	Slightly rounded	0.4	Probable	Chemoreceptor
Type g	Half proximal of 1st valvula	—	—	—	—	—
Campaniform	2/3 proximal of 1st valvula	—	Rounded	2	—	Mechanoreceptor
Styloconic 1	Proximal portion of 1st valvula	—	Rounded	0.7	No	Mechanoreceptor
Styloconic 2	Proximal portion of 1st valvula	—	Rounded	0.7	No	Mechanoreceptor
Styloconic 3	Proximal part, interior surface of 2nd valvulae	3	Slightly sharp	0.7	No	Mechanoreceptor

TABLE 3: Main biological features of *O. oryzivora* (host), *Platygaster diplosisae*, and *Aprostocetus procerae* (parasitoids). (According to Nacro and Nénon, 2006.)

	Nature	Average potential fecundity	Average fertility	Eggs' envelopes thickness	Length of the ovipositor	Distribution of the sensorial organs on the ovipositor	Nature of the parasitism
O. oryzivora	host	High (300 eggs)	Medium (35.6)	Thin (0.7 μm)	—	—	—
P. diplosisae	parasitoid	Very high	Not assessed	Very thin	40 μm	Mainly distributed on the distal portion of valve 3 Mechanoreceptors and chemioreceptors	Gregarious endoparasitism of the egg or L1 of the host
A. procerae	parasitoid	Low	Not assessed	Thin	1.2 μm	well distributed on the different parts of the ovipositor Mechanoreceptors and chemioreceptors	Solitary ectoparasitism of the pupae of the host

3.4. Sensillae on the Ovipositor of A. procerae. The paired 3rd valvulae bear four types of sensillae, three of which are trichoid sensillae.

(i) Type 1: a long trichoid sensillum, $104\,\mu m$ in length. It is located proximally (Figure 2(a)).

(ii) Type 2: this type of trichoid sensillum is the most common on the paired 3rd valvulae. These sensillae are nearly straight, $51\,\mu m$ in length and distributed on the 3rd paired valvulae up to $58\,\mu m$ from the base. They appear smooth when observed under the scanning electron microscope (Figure 2(b)).

(iii) Type 3: these are trichoid sensillae, $38\,\mu m$ in length, curved and observed from $42\,\mu m$ distally on the 3rd paired valvulae where 6 sensillae of this type are observed. They are channeled and slightly rounded distally (Figures 2(b), 2(c)).

(iv) Type A: this unique sensillum is observed at the proximal end of the 3rd paired valvulae. It is $5.3\,\mu m$ in length and originates from a depression. It is slightly curved and half of its proximal part is sharper than its basal part. This styloconic sensillum is the shortest sensillum of all sensillae observed on the 3rd paired valvulae (Figure 2(c)).

3.4.1. 1st Paired Valvulae. The 1st paired valvulae are very rich in sensillae. Eight types of sensillae were observed on these valvulae.

(i) Type b: the external process is located in a large groove. This type of sensillum is located in the proximal half of the 1st paired valvulae. It is basiconic (Figure 2(i)).

(ii) Type c: basiconic sensillum has a large groove. The external process is prominent, almost perpendicular to the axis of the valvula (Figure 2(j)).

(iii) Type d: styloconic sensillum, unique, with an appearance similar to a trichoid sensillum. It is located basally and is $4.7\,\mu m$ in length (Figure 2(k)).

(iv) Type e: a basiconic sensillum. Five sensillae of this type are arranged randomly, and the last one is located at $120\,\mu m$ distally from the base of the valvula (Figure 2(l)).

(v) Type f: 3 sensillae arranged in a triangle at the distal portion of the valvulae (Figure 2(m)). The external process is short. In their morphology and distribution, these sensillae look like the sensilla observed by Le Ralec [11] on the 1st paired valvulae of *Encarsia formosa* Gahen (Hymenoptera: Aphilinidae), larval parasitoid of the greenhouse Aleyrodid *Trialeurodes vaporariorum* Westwood (Homoptera: Aleyrididae).

(vi) Type g: the process is stretched and embedded in a depression (Figure 2(n)).

(vii) Campaniform sensillum: the external process is a dome. This sensillum is located on the basal 2/3 of the valvulae. Its diameter is $2\,\mu m$ (Figure 2(o)).

(viii) Styloconic sensillae of type 1 and type 2: one row of type 2 is enclosed by two rows of type 1. Type one has an external process that is entirely embedded in a depression. Three rows of styloconic sensillae are distributed on the external surface of the basic portion of the valvulae. They are $0.7\,\mu m$ in length (Figure 2(p)).

3.4.2. 2nd Paired Valvulae. The sensillae on the paired 2nd valvulae are less abundant than those of the paired 1st valvulae.

They include

(i) styloconic sensillae of type 3. Three of these sensillae are observed on the basal external surface of the valvulae. Their external process is more developed than that observed on the other types of styloconic sensillae. This process appears erect and oblique as compared to the axis of the valvula. The sensillum is embedded in a narrow depression. Its external process is $0.7\,\mu m$ in length (Figure 2(g));

(ii) sensillae of type g: they are located on the distal half of the valvula but are not illustrated.

The distribution of the sensillae on the valvulae of the ovipositor of *A. procerae* and their possible functions are summarized in the Table 2. The sensillae of the ovipositor of this eulophid are as abundant as diverse and probably mainly function as mechanoreceptors. The main biological features of the host (*O. oryzivora*) and its two parasitoids are presented in Table 3.

4. Discussion

Table 3 explains the main biological features of the host and its associated parasitoids. The reproductive biology of hymenopterans has been used to explain the nature of their parasitism. Price [12] showed that larval parasitoids of the wood fly, *Neodiprion swainei*, had a high fecundity and were gregarious. Their hosts were relatively abundant and easy to find. In contrast, the pupal parasitoids of the fly were ectoparasitoids with low fecundity. We are in a similar situation, where *P. diplosisae* and *A. procerae* share the same host at its different developmental stages due to the adaptations of their reproductive biology.

The ovipositor plays an essential role in the success of parasitism in Hymenoptera. Le Ralec [11], showed adaptive morphological features, according to the type of hosts, in 22 parasitoid hymenopteran species. These features are related not only to the morphology of the ovipositor (length and width of the diameter) but also to the quantity, the quality, and the way the sensillae are distributed on it. Thus, the species that easily access their hosts have ovipositors well equipped with mechanoreceptive sensillae spread along the length of the valvulae. The species that have difficulty accessing their hosts have poorly equipped ovipositors with mechanoreceptive sensillae that are generally grouped at the distal end of the valvulae. The case of these two parasitoid

species associated with *O. oryzivora* is consistent with what was stated above.

Indeed, we have already observed that most of the sensillae found on the ovipositor of *P. diplosisae* are mechanoreceptors located essentially at the extremity of the 3rd valvulae. These sensillae may be important for host selection, which for this parasitoid is either an egg or a larva. So, these sensillae could "inform" the parasitoid on the status of the surface of the host. The sensillae of type B, observed at the extremity of the 3rd valvulae, probably of chemoreceptor type, could "inform" the parasitoid on the interior status (parasitized or unparasitized) of the host. Lastly, the short length of the ovipositor (40 μm) seems an adaptation to this type of host where the thickness of the egg envelope is not more than 0.7 μm.

The ovipositor of *A. procerae* is not only well equipped with mechanoreceptive sensillae, but these sensillae are diverse and distributed along the length of the valvulae. In addition to these features, the length of the stylet of the ovipositor (1.2 mm) is 30 times the length of the ovipositor of *P. diplosisae*, and the 10 denticulations of the lancet meet the conditions of a parasitoid that exploits a host that is less accessible. As for *P. diplosisae*, the very abundant mechanoreceptive sensillae observed at the distal end of the paired 3rd valvulae could be used by *A. procerae* to detect the substrate within which the host is located and to determine the depth at which it is located. The three chemoreceptive sensillae of type F observed at the distal end of the paired 1st valvulae could "inform" the parasitoid on the depth and the condition of the host. The length of the ovipositor has already been recognized as an adaptive feature for several parasitoids that exploit the same host, *Tryporyza incertulas* Walker, a lepidoperan rice stemborer whose egg masses have different layers [13]. The two parasitoid species examined share the same resource by infesting different stages of the host and by the ovipositor of each species differing in length and associated sensillae. In fact, the parasitic action of the two parasitoids may be complimentary.

The role of the two examined parasitoids in the natural regulation of the AfRGM has already been investigated by several authors [1, 4–7]. These parasitoids parasitize the midge simultaneously, and they can find and kill up to 70% of the immature populations of the pest. However, sometimes, such a high level of parasitism occurs too late in the season to prevent large AfRGM populations from building up and causing serious yield losses. The role of these parasitoids could be integrated into an Integrated Pest Management (IPM) strategy that could include also cultural control (early and synchronized planting, management of alternative hosts and fertilizer), host plant resistance, and chemical control.

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Research Article

Concurrent Phenologies of Three Semiaquatic Bugs (Heteroptera: Gerridae, Veliidae) on a Small River in Central Illinois, USA

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The phenology of three species of Gerroidea (Heteroptera), *Metrobates hesperius* Uhler (Gerridae), *Rhagovelia oriander* Parshley (Veliidae), and *Rhematobates tenuipes* Meinert (Gerridae), was studied on a river in central Illinois (USA). *Metrobates hesperius* was the most abundant species, and was active from mid-May through mid-October. It was bivoltine and overwintered as eggs. Matinig and oviposition of *M. hesperius* were observed in mid-July. *Rhagovelia oriander* was present from mid-May to mid-November. This species was bivoltine (or possibly trivoltine), overwintering as eggs. *Rheumatobates tenuipes* was not active until early August, and was present to mid-November and was univoltine. It overwinters as adults and possibly as nymphs, and may undergo an extended early season diapause. The three species occupied differing microhabitats and differed in periods of peak abundance, with *M. hesperius* being most abundant from mid-May through the first of August, and *R. tenuipes* being most abundant from early August to mid-November.

1. Introduction

The families Gerridae and Veliidae belong to the superfamily Gerroidea [1], a group that usually inhabits the surface film in various microhabitats of lotic and lentic waters, and, in some instances, other terrestrial habitats with high humidity [2]. These animals function as predators and scavengers, typically feeding on other invertebrates trapped in the surface film [2, 3]. With the exception of a few of the larger species (e.g., *Aquarius remigis*) the biology and life history of many species have not been thoroughly investigated. In general, species of Gerroidea may be univoltine, bivoltine, or multivoltine [4], overwinter most typically as adults, and oviposit on or in substrates closely tied to their epipleustonic habitat. A number of species exhibit different patterns of wing polymorphism, with macropterous forms dispersing to other habitat patches [3, 5–7]. In addition to hibernation, some species also undergo diapause, most often in the adult stage (e.g., [3]).

While individual North American gerroid taxa have been the subject of numerous life history studies [4], and several assemblages (*sensu* [8]) have been examined (e.g., [9–13]), life histories of gerromorphans on rivers are rare, perhaps due to the greater difficulty of sampling these habitats relative to streams and ponds.

I examined Gerroidea species occurring on the Sangamon River in central Illinois, (USA) where the numerically dominant taxa are *Metrobates hesperius* Uhler, *Rhematobates tenuipes* Meinert (both Gerridae), and *Rhagovelia oriander* Parshley (Veliidae). At the study site in Mahomet, Illinois, the Sangamon River drains an area of 93.200 ha, with a base hourly discharge of less than $5\,\mathrm{m^3 s^{-1}}$, but peak discharge in flood events commonly greater than $20\,\mathrm{m^3 s^{-1}}$ and occasionally greater than $80\,\mathrm{m^3 s^{-1}}$ [14]. Tree cover at and near the shoreline is dominanted by *Acer saccharinum* L. (silver maple), *Fraxinus pennsylvanica* Marsh. (green ash), and *Platanus occidentalis* L. (sycamore) [15, 16].

2. Methods

All sampling was at the Sangamon River at US Highway 150 in Mahomet, Champaign County, Illinois, USA (40.1919° N,

88.3998°W, elevation 207 m a.s.l.). Sampling was conducted February 23, April 2, and approximately weekly (every 6–8 days) from April 25 through November 10, 2000, with one week missed in late June and another missed in early October. The river was too large and deep to effectively sample by wading, and fallen trees, shallow areas, and rocks precluded use of a motorboat. Therefore, sampling was conducted from a whitewater kayak. Sampling was qualitative, consisting of collecting representative samples of all epipleustonic taxa using an aquarium net, with an opening of 15.2 × 10.2 cm. Samples were collected into 70–80% ethanol.

In the laboratory, specimens of *M. hesperius*, *R. tenuipes*, and *R. oriander*—the three dominant gerroids at this site—were sorted to developmental stage on the basis of body measurements, and larger individuals were sexed. Measurements were made using an Olympus SZH 10 or SZ 60 dissecting microscope with a calibrated ocular micrometer. Measurements included width across eyes and lengths of the pro-, meso-, and metafemora.

Pairwise plotting of the four metrics revealed six distinct size classes of individuals, corresponding to the five nymphal stadia and adults, for each species. These data were then plotted to assess phenology of each of the three species. For all figures, tick marks on top axis (below months) indicate sampling dates. Voucher specimens have been deposited in the INHS Entomology Collections.

3. Results

Metrobates hesperius was present from mid-May through mid-October (Figures 1 and 2). First instars were present from mid-May to mid-June, and again in late July and mid-August; second instars, from early June through early July and in early and mid-August; third instars, from mid-June through early July and then from late July through mid-August, with a small number of individuals in mid-September; fourth instars, from mid-June through mid-August, with fewer in mid-July through early August, suggesting two peaks of abundance; and fifth instars from mid-June through mid-August, and again in late September.

Adults appeared in early July and were present through mid-October. All adults were apterous. I observed adults depositing eggs on the lower surface of floating *P. occidentalis* and *A. saccharinum* leaves on 11 July, and 46 eggs on one *P. occidentalis* leaf were collected on that date. Adults were observed aggregating on at least 10 leaves (both green (fresh) and brown (decaying)) floating down current, aggregations numbering 5 to 15 individuals, apparently with males mating, or attempting to mate, with females, and females also ovipositing. They also aggregated in a similar manner around floating bits of bark.

Rhagovelia oriander was present from mid-May through the end of sampling in mid-November (Figures 3 and 4). First instars were present in mid- and late May and in mid-July; second instars, in mid-May and mid-August; third instars, in early June and late July; fourth instars, in early and from mid-June through early August; fifth instars,

FIGURE 1: Percent of individuals of *Metrobates hesperius* in each stage on each sample date.
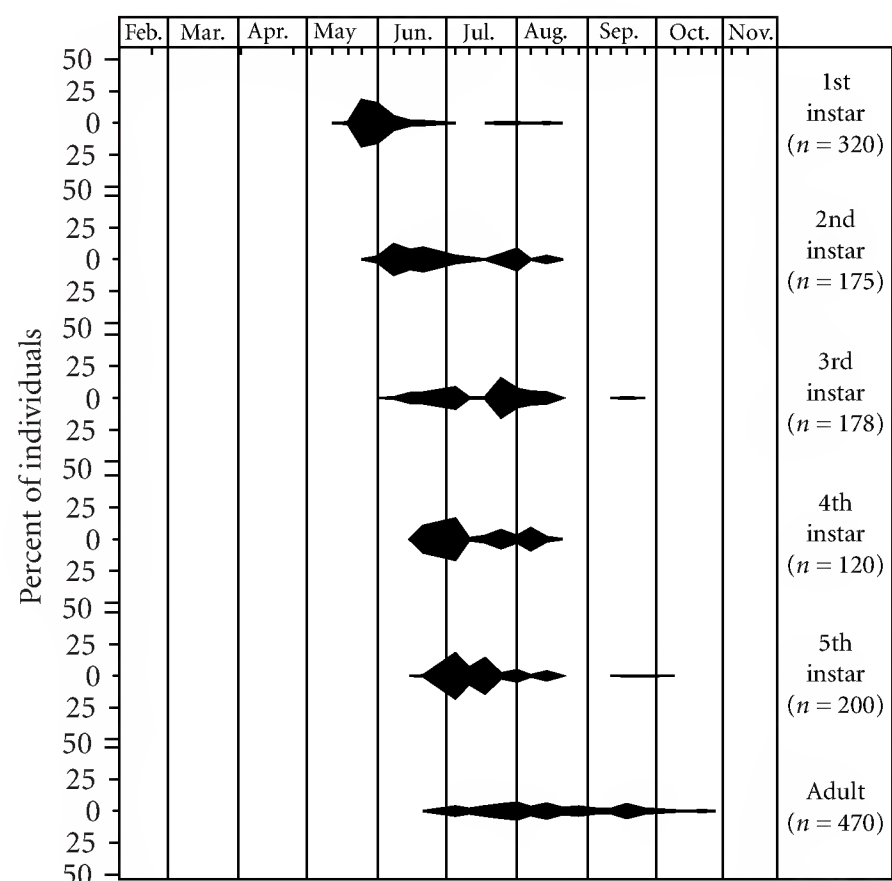
FIGURE 2: Percent of individuals of *Metrobates hesperius* of same stage across all sample dates.

from early to mid-June, mid-July to mid-August, and in mid-September. Although adults were present continuously from mid-June through mid-November, a peak from mid-June through mid-July is easily distinguishable from the later season adults. Only one macropterous individual was collected (21 October, an adult female), the remaining adults were apterous.

Rheumatobates tenuipes was present from early August through the end of sampling in mid-November (Figures 5 and 6). First instars were present in early and late August; second instars, from early through late August; third instars,

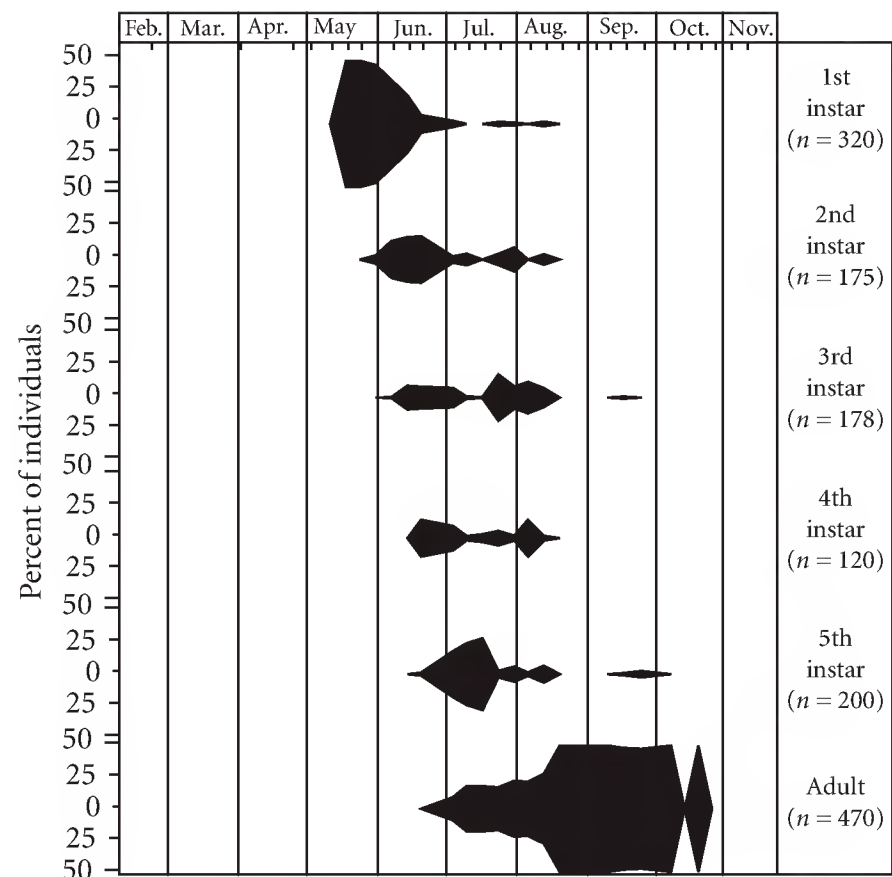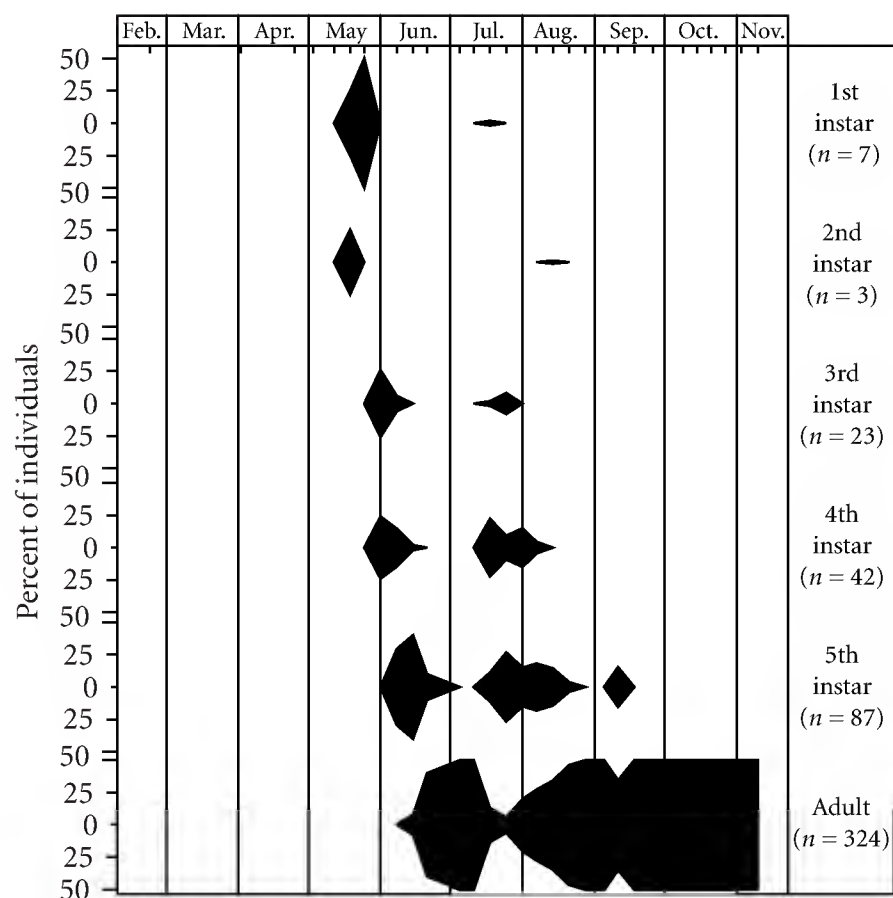FIGURE 3: Percent of individuals of *Rhagovelia oriander* in each stage on each sample date.
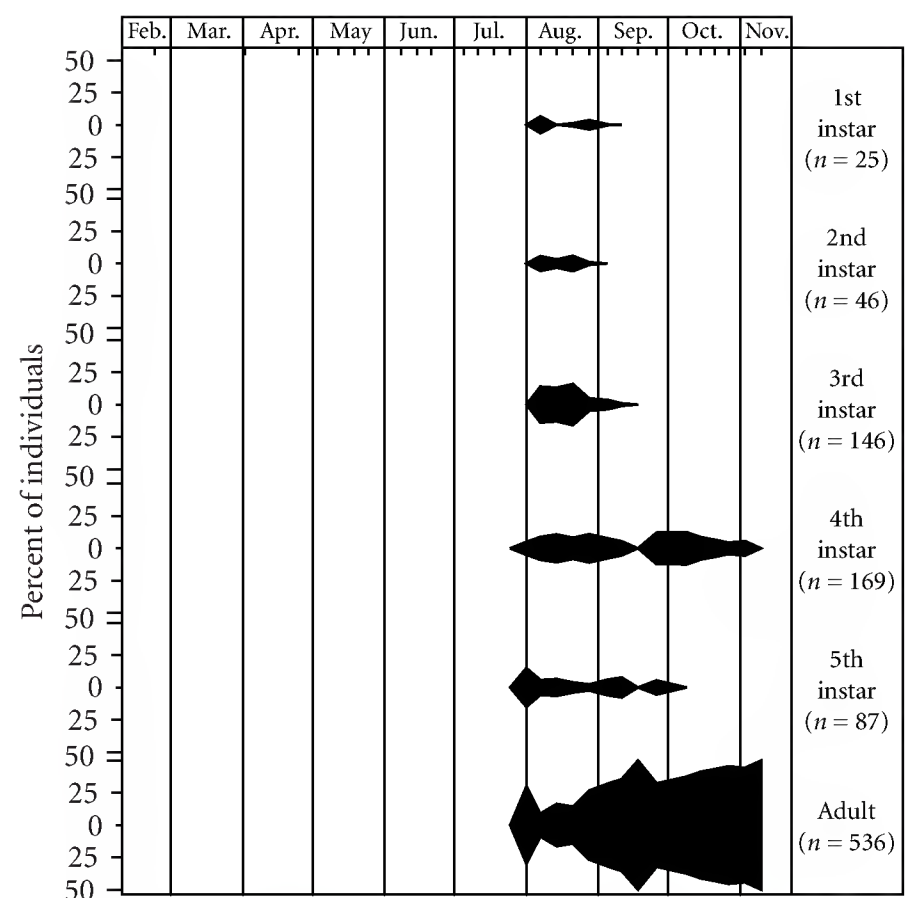
FIGURE 5: Percent of individuals of *Rheumatobates tenuipes* in each stage on each sample date.
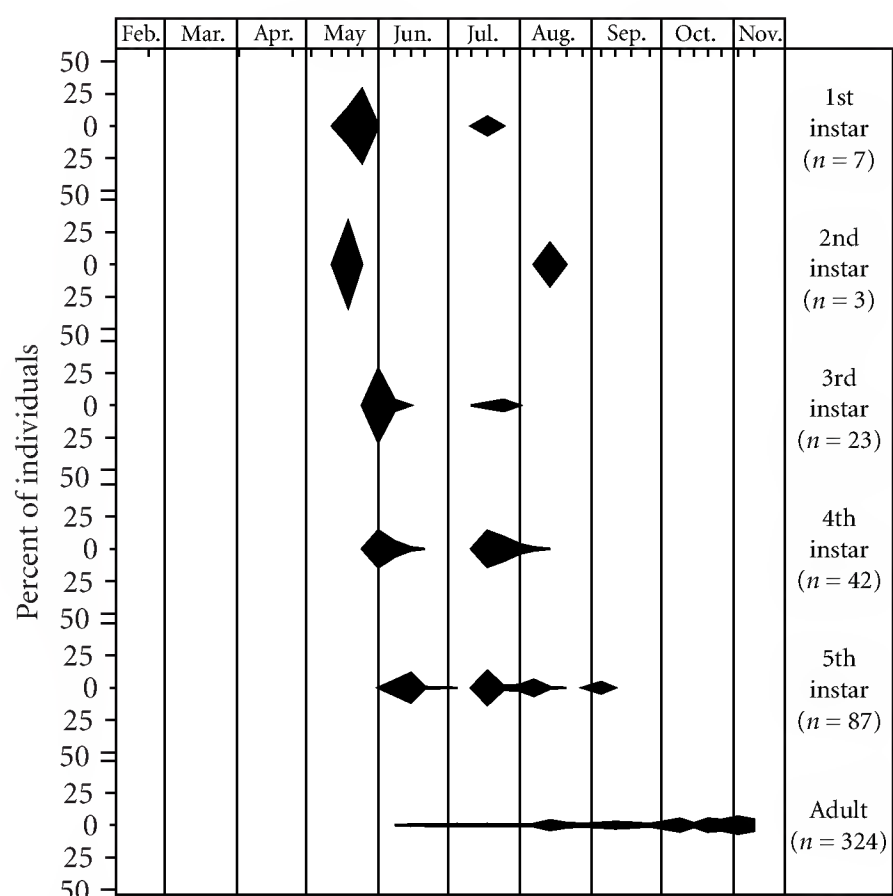
FIGURE 4: Percent of individuals of *Rhagovelia oriander* of same stage across all sample dates.
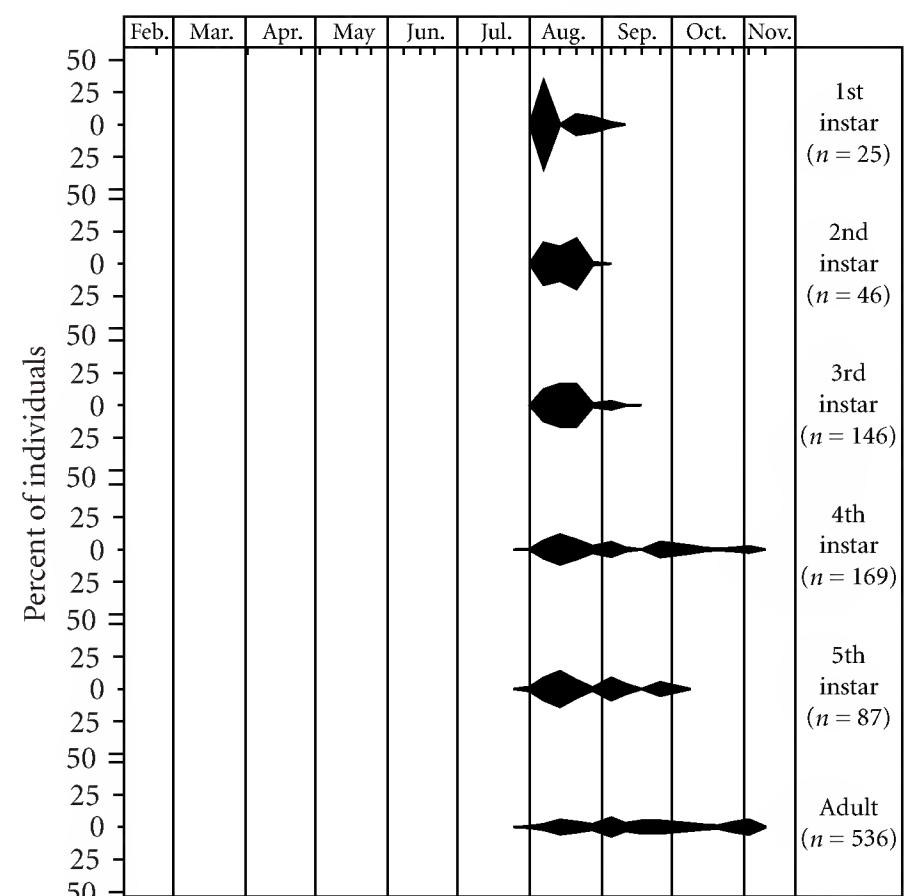
FIGURE 6: Percent of individuals of *Rheumatobates tenuipes* of same stage across all sample dates.

from early August through mid-September; fourth instars, from early August through mid-September, with a second peak from late August through early November; fifth instars were present from early August through mid-September and in late September. Adults were present from early August through mid-November, and were present a week before first-third instars were recorded. All adults were apterous, except for one female collected on August 7, and three females collected on August 14. Several adult females

collected on these two dates were visibly hypogastric (August 7, $n = 8$; August 14, $n = 6$), indicating that they were about to oviposit.

From early August through mid-October, all three species were active concurrently (Figures 7 and 8). *Metrobates hesperius* was the numerically dominant gerromorphan on the river from mid-May through the first of August (Figures 7 and 8). From early August to nearly the end of sampling, *R. tenuipes* was numerically dominant. *Rhagovelia oriander*
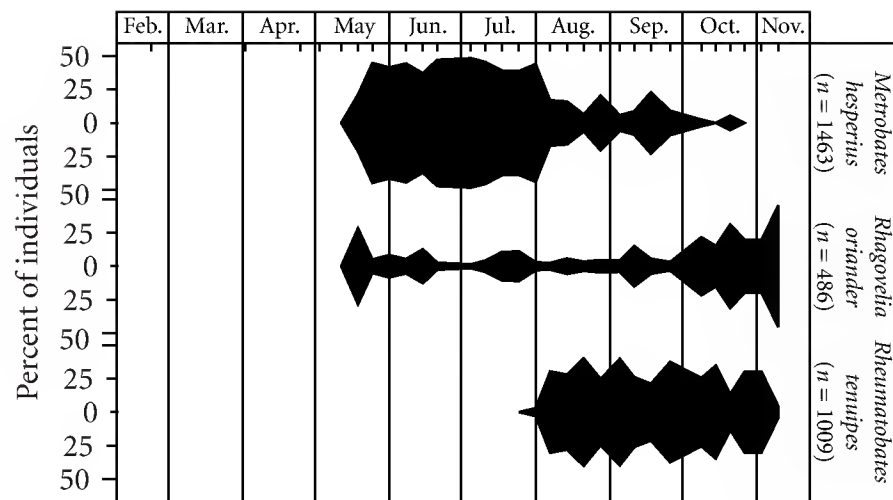
FIGURE 7: Percent of total individuals of each species (*Metrobates hesperius*, *Rhagovelia oriander*, and *Rheumatobates tenuipes*) on each sample date.
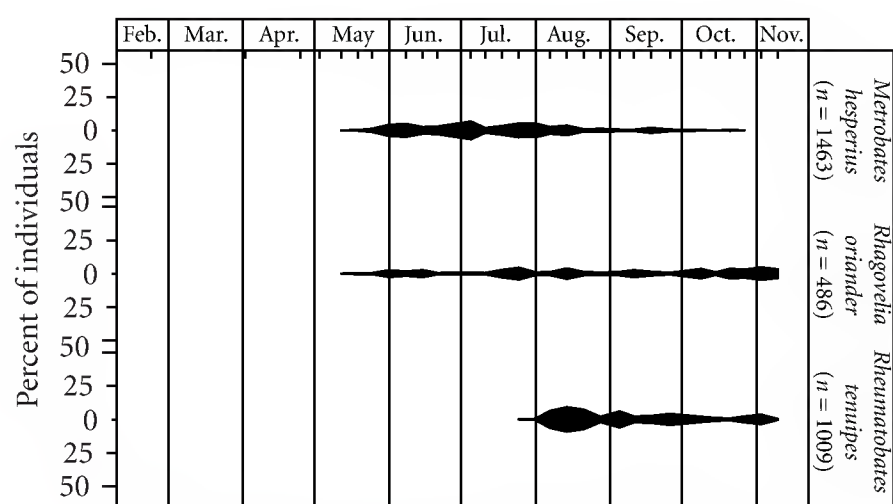
FIGURE 8: Percent of total individuals of each species (*Metrobates hesperius*, *Rhagovelia oriander*, and *Rheumatobates tenuipes*) across all sample dates.

became increasingly abundant beginning in early October, and remained abundant through the end of sampling in mid-November.

Metrobates hesperius was generally observed in large numbers on open, visibly flowing portions of the river. In July, adults appeared to aggregate in shadows cast by overhanging trees, rather than in direct sunlight, moving to well-lit areas only when approached (in a kayak), and gradually reassembling in the shade following such disturbances. *Rhagovelia oriander* was always found just downstream of obstructions (fallen logs, limbs, rocks) in association with eddies adjacent to rapidly flowing water. *Rheumatobates tenuipes* was found primarily along shaded banks, with little or no detectable flow. At low water levels, banks were undercut with many exposed tree roots, and, under these conditions, *R. tenuipes* was commonly found on the water surface up under the banks of the river.

4. Discussion

Metrobates hesperius is bivoltine in central Illinois, with a possible much smaller third generation. The present data suggest that this species overwinters as eggs. The seasonal occurrence of *M. hesperius* in the present study also is consistent with earlier observations [17–21], although in southeastern Louisiana Ellis [22] reported collections

(stage not indicated) as late as November. Oviposition on organic materials floating down river in the current could function as a means of dispersal in the egg stage.

Rhagovelia oriander was found from May into November in central Illinois, which is generally consistent with previously reported seasonal data [23–25], although Bacon [23] also reported apterous males in April. This species is bivoltine in central Illinois, overwintering in the egg stage. From mid-September through mid-November, only adults of *R. oriander* were present. Unlike the European *Velia caprai* Tamanini, which can overwinter as both eggs and adults [26], *R. oriander* appears to overwinter exclusively in the egg stage.

Reumatobates tenuipes appears to be univoltine in central Illinois, overwintering as adults and possibly as nymphs, but the September to November peak of fourth instars suggests there could be a second generation, with early instars missed in sampling. I found *R. tenuipes* from early August to early November, and this is consistent with most literature records, which are primarily from September and October [18, 20]. However, Bobb [27] reported nymphs and adults somewhat earlier in Virginia, in early June. The status of *R. tenuipes* earlier in the season is unclear, but it is possible that the species is in summer diapause (aestivation) during April–July, or that its appearance in August could represent immigration from other habitats. The presence of fourth and fifth instar nymphs in early August, however, suggests immigration does not account for the observed pattern.

All three of the Gerroidea species in this study (*M. hesperius*, *R. tenuipes*, and *R. oriander*) commonly have been reported from large streams and rivers [17–21, 24, 25, 27–29]. While several studies have reported these species together in the same habitats [12, 13, 17], their cooccurrence likely depends on a variety of interacting factors (e.g., [3, 13, 30]).

This study fills a gap in the literature by providing data on concurrent phenologies of these cooccurring species. These data provide an example in which, when the three species cooccur, the taxa appear to partition the small river habitat both in space—position on river—and time, with differing seasonal periods of peaks abundance. Such differences in seasonality and microhabitat might relate to interspecific interactions such as competition or to differing adaptations to the environment [31]. Whether these cooccuring Gerroidea species have adapted to coexist in response to intraspecific interactions or as differing adaptations to the physical environment remains open to study by future workers interested in phenologies and life history strategies of these species.

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Research Article

Functional Morphology of Secretion by the Large Wax Glands (Sensilla Sagittiformia) Involved in Tick Defense

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Ticks are protected against ants by release of an allomonal defense secretion from the large wax glands (or type 2 glands) that line their bodies. To explore how the large wax glands operate, before and after microscopic observations of these glands (nonsecreted versus secreted test groups), mass determinations were made for *Rhipicephalus sanguineus* that had been exhausted of secretion by repeated leg pinching to simulate attack by a predator. Prior to secretion, the glandular organ is fully intact histologically and matches the *sensillum sagittiforme*, a key taxonomic structure described in the 1940s. The large wax gland is innervated and responds to pressure stimulation as a proprioceptor that stimulates the secretory response. Histological observations after secretion has occurred show that the entire glandular contents and associated cells are jettisoned out of the gland like a syringe. The glandular cellular components are subsequently rebuilt by underlying hypodermal cells within a few days so that secretion can take place again. Presumably, the active allomonal ingredients (hydrocarbons) are released when these derived epidermal cells reach and burst onto the cuticular surface. Our conclusion is that the large wax glands are holocrine and feature intermittent regeneration.

1. Introduction

This article updates the 1949 *Psyche* paper of Dinnik and Zumpt's [1] that reported on the tick's secretory capacity and structure of the sensilla sagittiformia (= arrow-organs, [2], Figure 1 bottom left) that are now known as large wax glands [3] or type 2 glands [4]. These glands are dermal and present largely on dorsolateral surfaces of Metastriate ticks (include the majority of hard ticks of medical-veterinary importance except *Ixodes*) and they are considered key species-specific taxonomic characters [5, 6] for ticks in this lineage. The most notable function of the emission from these glands is its role in defense (allomone) and protection against ants [7, 8]. The secretion has also been shown to have strong antimicrobial activity [8, 9]. The secretion's mode of action in defense agrees with the majority of antiant allomones in insects [10]. The allomone produced by the large wax glands is rich in hydrocarbons [3] that presumably block the ant's antennal chemosensory receptors so that the tick is not recognized or neutralizes ant aggressiveness that effectively hides the ticks from the ants. This nonirritating allomone is directed specifically toward ants and no other predator [11, 12]. One of the major components of the secretion is squalene [3] that is sequestered from the host bloodmeal (not in larvae as they have not yet fed on blood [13]). Squalene has been shown to modify tick behavior by acting as an arrestant [14, 15]. Consistent with the arrestant function, ticks cluster around and on conspecific ticks that have secreted or aggregate on surfaces treated with the squalene secretions producing highly species-specific aggregations [16], thus suggesting that this secretion has a pheromonal role (synonmy of glands/pheromone associations are given in Walker et al. [4]). In Schulze's 1942 [2] initial description, he commented on the ability by these integumental organules (large wax glands) to secrete, speculating that the fluid kept the tick from drying out and was involved in chemical communication. This secretion generates no extra waterproofing to enhance water conservation by limiting cuticular permeability [17], but it is clear that this secretion from the large wax glands modifies tick behavior and has a definite semiochemical function.

Pressure stimulation, leg pinching that simulates attack [3] or force from blood feeding [4, 18], prompts the large wax glands to activate and release copious amounts of secretion that appear as bursts of droplets that exude along the edges of the tick's body. The secretion evaporates as it spreads over the body, giving the tick a residual sheen. Once blown, these glands in adult ticks gradually reload (immatures have not been examined) and regain full secretory capacity after approximately 10 days [3]. Evidence of neural control of the large wax gland is apparent from the observation that a burst of secretory activity is regional and occurs onto the cuticle that is just above the leg that is being pinched [2, 3]. Light pinching of one or several legs elicits a small pulse of secretion [7], while forceful stimulation of the entire tick (or immersion in organic solvent) causes all large wax glands to discharge their secretion simultaneously [19]. Thus, the amount of secretion corresponds with the intensity of the stimulus. Killed ticks do not secrete [3]. All Metastriate tick stages (larvae, nymphs, and adults) have large wax glands [1, 2, 6]. Furthermore, these glands secrete in all stages [3, 19], confirming a highly conserved, innervated glandular morphology that spans the life history of the tick as figured by Dinnik and Zumpt [1]. The morphological classification of these large wax glands has yet to be determined histologically. The goal of this study was to explore the mechanics and microscopic anatomy of how the large wax gland secretes and to account for its ability to secrete again after being depleted. We used brown dog ticks (kennel tick) *R. sanguineus* (Latreille) to parallel observations made by Dinnik and Zumpt [1].

2. Materials and Methods

2.1. Induction of Secretion from Ticks. Larvae, nymphs, and male and female adults of *R. sanguineus* were obtained from established laboratory colonies maintained at Oklahoma State University using rabbits (*Oryctolagus cuniculus* L.) as the host for larvae and sheep [*Ovis aries* (L.)] as hosts for nymphs and adults; IACUC Protocol number AG50212, Oklahoma State University, Stillwater, Okla, USA, exp. 2 May 2010. Transfer of ticks was accomplished with an aspirator. Only nonfed ticks were used in this study, and all ticks were about 2 months of age after ecdysis or hatching. In our laboratory, ticks were stored in environmental cabinets (Fisher Scientific, Philadelphia, Pa, USA) in 3000cc (L × W × H) glass desiccators at 93% RH (±SE < 2%RH); saturated KNO$_3$ at base of desiccator; [20], 25°C (±SE < 1.0°C) and 15 h : 9 h L : D. The 93% RH is above the critical equilibrium humidity for all stages of this tick [21]. All ticks were in healthy condition and displayed regular ambulatory activity.

Ticks were induced to secrete by placing them onto the stage of a stereomicroscope (40x) and gently pinching their legs with forceps, which caused the formation of visible secretory droplets on the surface of the ticks. Larvae were treated similarly except they were anchored onto a piece of double-sided tape that was mounted on the stage of the microscope to permit observation of their secretions. When the tick's legs are pinched, typically all legs extend outward,

droplets of fluid exuded out the sides of the tick's body, then the tick curls its legs under its body, and the fluid spreads over the tick's body as it evaporates. The tick remains motionless, with the legs retracted, as though playing dead, then about 30 seconds to one minute later, the tick uncurls its legs and crawls rapidly in a seemingly flight reaction [3, 7]. To obtain ticks that did not secrete, and to act as controls, ticks were permitted to crawl onto a soft camel's hair brush, examined under the microscope while still on the brush (for absence of droplets, sheen, and behavior associated with having secreted), transferred to 1cc glass vials and then frozen at −10°C for 3 hours and then thawed to room temperature. A closed system of HCN vapor was also used as an alternate killing method.

2.2. Determination of Time to Replenish Secretory Reserves. Ticks were weighed using an electrobalance (SD ± 0.2 μg precision and ±6 μg accuracy at 1 mg based on five mass measurements of a 1 mg weight at the 200 mg range; Cahn, Ventron Co., Cerritos, Calif, USA). Ticks were weighed individually, without enclosure or anesthesia, and were transferred by lifting the tick to the weighing pan using a soft camel's hair brush. Weighing of a tick took place in less than 1 minute. In this experiment, ticks were stimulated to exhaustion (secretion was no longer produced) and then reweighed after pinching their legs again until they lost the same amount of secretion at initial stimulation [3]. Briefly, a tick was weighed, placed on the stage of a microscope, induced to secrete by pinching the legs with forceps until it could no longer secrete (typically less than 10 seconds), and then the tick was reweighed. Mass loss after stimulating the tick was primarily considered to be lost from secretions because of the short time to exhaust the ticks. The amount of mass that the tick lost was expressed as a percentage: percentage changes in mass $= 100(w_t − w_i)w_i$, where w_t is the mass after the tick was stimulated to exhaustion at any time t, and w_i is the initial mass of the tick before its secretory reserves were exhausted. Experimental conditions were held constant at 93% RH, 15 h : 9 h L : D, and 25°C. Every two days after initial secretion, groups of ticks were restimulated to exhaustion and reweighed, and the time to replenish was taken as the time when the ticks emitted the same amount of material as the initial stimulation; this was conducted over a time period of 2 weeks.

The experiment was replicated three times using 10 ticks at each time point (total $N = 30$ ticks at each time point) with each replicate coming from a separate rearing batch of ticks. Percentage mass change data were compared using analysis of variance (ANOVA) using an arcsin transformation for percentages [22]. Killed ticks served as controls. All killed ticks were used in freshly killed condition; that is, they had not been dead for more than 2 hours. In this experiment, nonfed adult females, nonfed adult males, nonfed nymphs, and unfed larvae were tested.

2.3. Preparation of Tissue for Microscopic Examination. Twenty nonfed female adult ticks were placed into two groups (ticks came from three separate rearing batches).

Preparation of tissue was done following a technique modified from vertebrate histology [23, 24]. The nonsecreting group of ten ticks (described above) was quickly placed in cold (8°C) Trump's fixative and incubated for one hour at 8°C (Electron Microscopy Sciences, Hatfield, Pa, USA). The other group of ten ticks was induced to secrete (described above) and then immediately placed into cold Trump's fixative and stored under refrigeration (8°C). After an initial hour of fixation both groups of ticks were then cut with a razor blade midsagittally and placed back into fresh Trump's fixative and shaken with a tissue agitator at 8°C for 24 hours. We emphasize that the ticks that were induced to secrete were not previously killed, thus, the changes in glandular morphology are a result of secretion and not due to freezing. In fact, large wax glands from ticks that were killed by freezing (ticks were killed so as not to activate secretion by chemical treatments during tissue preparation [3, 19]) showed exact intact morphology as ticks killed with HCN (data not shown).

The tick midsagittal pieces were trimmed under a dissecting scope (40x) parasagittally until only 3 mm of the most lateral cuticle were present. These small sagittal sections of the body wall were dehydrated using a graded series of ethanol (70%, 85%, 2 × 95%, 2 × 100%; Sigma Chemical Co., St Louis, Mo, USA). Sections were then incubated in a 1 : 2 and 1 : 1 Spurr's plastic (Electron Microscopy Sciences, Hatfield, Pa, USA): 100% ethanol for 30 minutes each before being infiltrated in pure Spurr's plastic overnight. Next, samples were embedded and cured in fresh Spurr's plastic at 60°C for 48 hours in an isotemperature vacuum oven (Fisher). Using a dry glass knife and an LKB-Ultramicrotome III (LKB-Ultrotome III, LKB-Products, Produkter AB, Bromma, Sweden, Europe), serial sections (2-3 μm) were cut from plastic blocks and placed on standard microscope slides. Typically 25 serial frontal sections of the body wall were made for each tick, resulting in microscopic examination of a total of approximately 250 serial sections for each group. Thus, total observations from both groups of ticks equal 500 serial sections that represent three separate rearing batches. The integumental glands were visualized within the cuticle using a basic fuchsin and toluidine blue composite stain [25].

All integumental sections were examined using an Olympus compound light microscope (Olympus America, Center Valley, Pa, USA) to ascertain the microscopic anatomy of the glands present within the lateral cuticle. Photographic images were taken at various magnifications using a SPOT digital camera (Diagnostic Systems Laboratories, Webster, Tex, USA), and plates were constructed using Adobe Photoshop CS (Adobe Systems, San Jose, Calif, USA).

3. Results

3.1. Anatomy of the Large Wax Gland.
Considerable numbers of large wax glands are located within the most lateral integument along the entire body wall of female adults of R. sanguineus. Most of the distal arrow-shaped duct of the gland, along with the pore, tuft chamber, and terminal chamber lie in a frontal plane in reference to the body axis and are entirely encased within the hard chitinous portion of the integument. Figure 1 bottom right represents a schematic drawing of the entire large wax gland found in R. sanguineus. The corresponding 1942 drawing redrawn from Schulze [2] is in Figure 1 bottom left. The most conspicuous part of the gland is the distal arrow-shaped duct (Figure 1, D) that empties through a large pore (Figure 1, top, Po; Figure 2, insets) onto the surface of the cuticle. The distal duct communicates proximally through a small-centralized opening within the floor or base (Figure 1, V) of the arrow-shaped duct (Figure 1, bottom left and right). The middle chamber of the organ called the tuft chamber is almost entirely occupied by a structure resembling a "gas-flame," "flame-cell," or a "tuft of hair" (terms used by Schulze [2]) that is known as the tuft (Figure 1, T). The base of the distal duct is heavily sclerotized and appears to act as a boundary or as valve arms that limits entry into the juxtapositioned tuft chamber (Figure 1, Tc) below. The tuft appears to be nonchitinous and transparent. The surrounding tissue just under the distal arrow-shaped duct and in close association to the middle tuft chamber and the terminal/glandular chamber appears spongy in its consistency and seems to have many elastic elements within its matrix (Figure 1, S); see Figure 2(a). This elastic material continues distally and surrounds the entire gland and makes up the boundaries/frame of the pore of the gland that opens to the body surface (Figure 1, top: B, F; Figure 2(a), insets).

The tuft in the middle chamber of the gland attaches to the robust scolopale (cuticular sheath around end of dendrite) via a filament (Figure 1, Fa) that extends into the distal portion of the terminal chamber and terminates at its meeting point with the axial fiber of sensory neurons (Figure 1, A). Where the filament of the scolopale encounters the axial fiber (Figure 1, K), a swelling occurs that is known as the knot of the scolopale [2]. The distal portion of the terminal chamber (Figure 1, Pc) lumen remains open and is occupied by the filament of the scolopale, the knot of the scolopale, the axial fiber, and the lateral projecting edges of the scolopale (Figure 1, P). The proximal lumen of the terminal chamber (Figure 1, Pc) is almost completely filled by cytoplasmic processes of the glandular cells (Figure 1, G) laterally and medially by the centralized axial fiber (Figure 1, A) that presumably extends down into the deepest recesses of the terminal chamber where it merges with sensory nerve nuclei. These neuronal cell nuclei are not visible (Figure 2(a)) in any of our sections as they are enveloped by the larger glandular cells that extend past the terminal chamber of the gland and into the actual body cavity.

3.2. Large Wax Gland before and after Secretion.
Figure 2(a) shows a typical large wax gland within the integument of the ticks that were treated by freezing for three hours. The gland is completely intact and shows regular morphology as described in Figure 1. Results were similar for ticks killed with HCN. The dark-staining chitinized floor of the distal arrow-shaped duct is almost completely closed with just the tip of the tuft penetrating up into the distal duct lumen from the tuft chamber. The lips of the surface pore are constricted
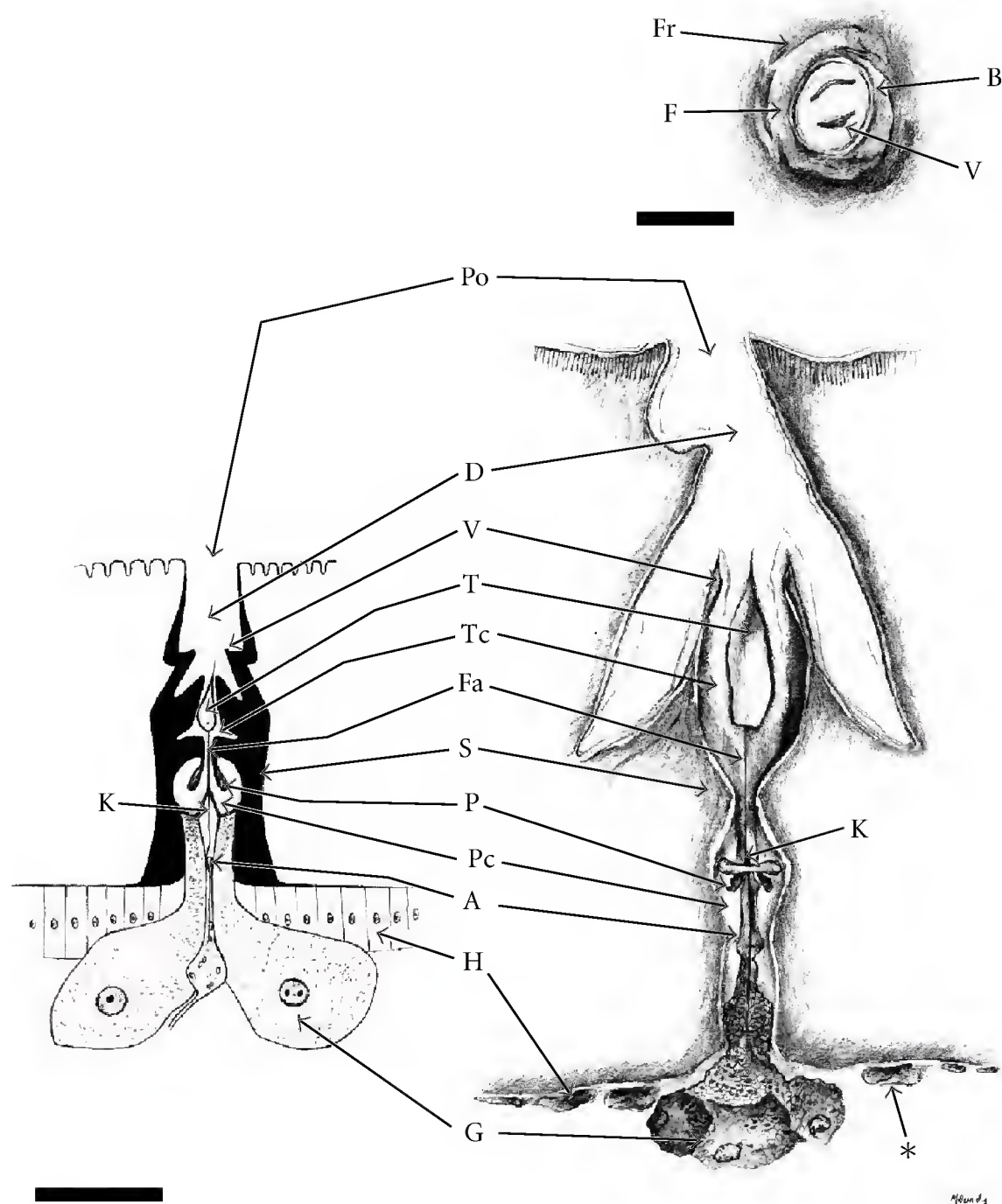
FIGURE 1: Representative drawings of the large wax glands (sensilla sagittiformia = arrow-organs) in nonfed adult females of *R. sanguineus*. Top: direct view looking down on the pore opening of the gland. Bar = 10 μm. This transverse view of the gland opening shows the fringe (Fr), the spongy elastic chitinous frame (F), the border of the pore opening (B), and the top of the valvular arm (V), that make up the roof of the tuft chamber. Bottom left: redrawing of the original 1942 description of the *sensillum sagittiforme* in sagittal view from *Hyalomma marginatum* Koch by Schulze [2] (also redrawn in Dinnik and Zumpt [1]). Bottom right: drawing of the large wax gland from the present study (redrawn from Figure 2(a)). Labeled structures: pore opening of gland (Po), distal arrow-shaped duct (D), valvular arm of the roof of the tuft chamber (V), tuft (T), tuft chamber (Tc), anchoring filament of the tuft (Fa), knot of the scolopale (K), spongy elastic chitin (S), projecting edges of the scolopale (P), proximal terminal chamber (Pc), axial sensory nerve fiber (A), hypodermis (H), glandular cells (G), and enlarged juxtapositioned transdifferentiating epithelial cells (∗). Bar = 30 μm.

when looking down on the sagittal view of the gland pore opening (10 μm) (Figure 2(a), inset) that is half the size of the pore opening (22 μm) in the exhausted glandular organ (Figure 2(b), inset). Also, it is important to note that the right and left arms that make up both the floor of the distal duct and the roof of the tuft chamber are seen in transverse section inside of the pore opening (Figure 1: top, V; Figure 2(a), inset). The tuft, the entire scolopale, the axial fiber, and the glandular cells are all intact within the gland organ.

Ticks that were pinched on the legs with forceps causing release of secretion present a completely different morphology to their large wax glands (Figure 2(b)) than those that had not secreted. Most of the large wax gland's internal morphology has been destroyed. The lumina of the distal duct, tuft chamber, and terminal chamber appear to be greatly widened compared to the large wax glands that have not released their secretions. The tuft, some of

the scolopale, the axial fiber, and the glandular cells are absent in sagittal section within exhausted large wax glands. Cytoplasmic pieces of the glandular cells are seen within the extended tuft chamber, distal duct lumen (Figure 2(b), black arrow), and on the surface of the cuticle. The chitinized arms of the floor of the arrow-shaped duct have been forced open and disappear from view within the transverse section of the pore lumen (Figure 2(b), inset). The pore lumen is also packed with broken cytoplasmic pieces of the glandular cells. The wall of the scolopale and both projecting edges are damaged but intact within the sagittal view of the exhausted organs. The spongy elastic integument surrounding the large wax gland's terminal chamber has been pushed up and thinned out in response to the widening of this proximal most chamber. The result of this relocation of the spongy elastic chitin distally has lead to an increase in this boundary material (25 μm thick) surrounding the
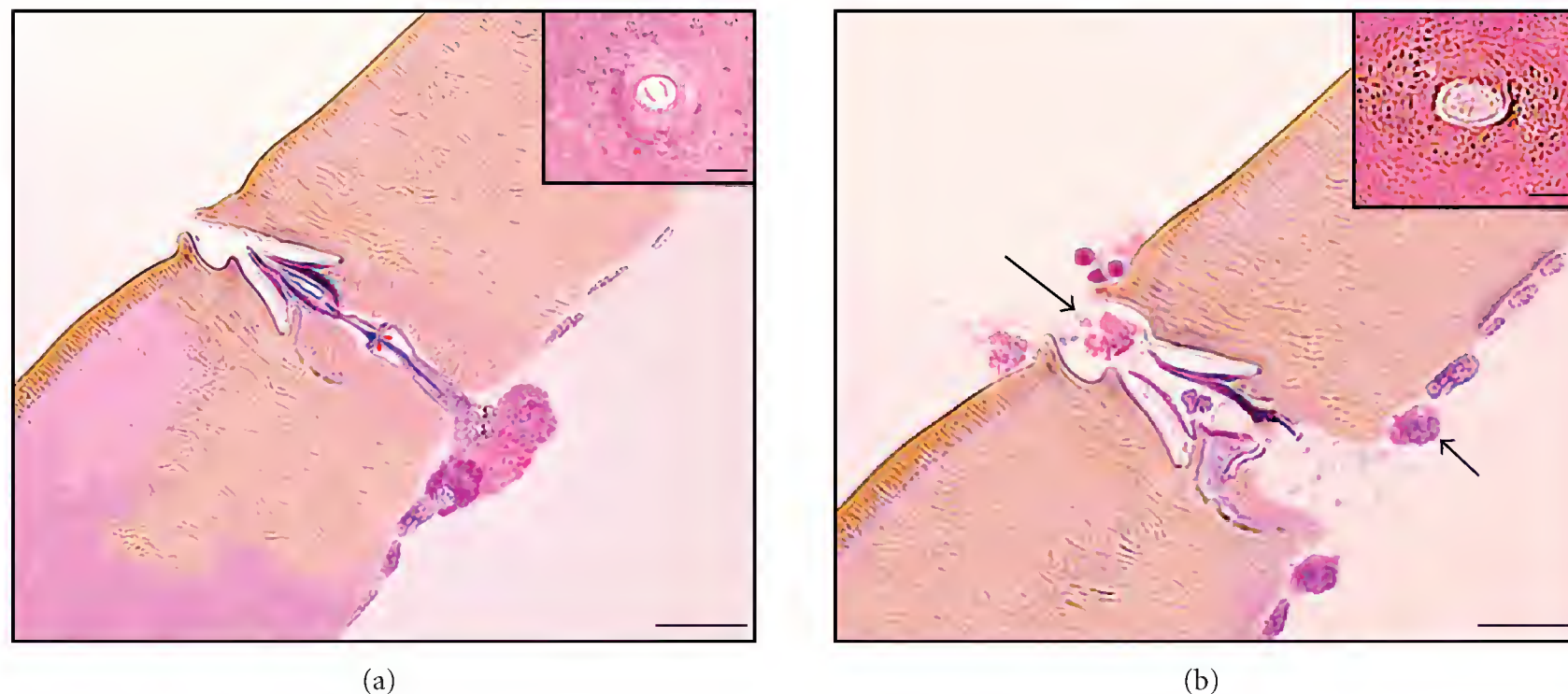
(a)

(b)

FIGURE 2: Micrographs of sagittal sections of large wax glands (sensilla sagittiformia = arrow-organs) in nonfed adult females of *R. sanguineus*. (a) Intact large wax gland showing regular microscopic anatomy (corresponding drawing in Figure 1 with parts labeled that match Schulze's [2] description). The gland has a restricted pore opening (inset, direct view), a thick spongy chitinous layer, and three large glandular cells occupying most of the proximal terminal chamber lumen. Bar = 30μm, and Bar = 10μm (inset). (b) Evacuated large wax gland of ticks that had been stimulated to secrete. Most of the morphology of the large wax gland has been destroyed. Glandular cells have been forced up into the tuft chamber and distal duct, and as they existed under high pressure these cells have been broken into pieces (long arrow). The glandular cell pieces have forced open the pore and widened its diameter and the spongy elastic chitinous frame around the gland pore (inset). Note that juxtapositioned cells are hypertrophied and have started the differentiation process to replace glandular cells that had been ejected during release of the secretion (short arrow). Similar morphology was observed in 20 replicates, and these two micrographs are representative of those replicates. Bar = 30μm, and Bar = 10μm (inset).

widened pore (Figure 2(b), inset) when compared to the boundary material (13μm thick) around the regular pore opening from an unstimulated large wax gland (Figure 2(a), inset).

Cells within the hypodermis that are in juxtaposition to these glands (Figure 2(b), white arrow) are more cuboidal in shape than the regular squamous shape of the cells that make up the majority of this epithelium. These hypodermal cells appear to have a change in cytoplasmic morphology as they mature. Most notably, these cells begin to accumulate granules within their cytoplasm as seen in Figure 2(b). These hypodermic granulated cell types, which are in close proximity to the terminal chambers, have been observed in both test groups. These morphological changes that were described for the large wax glands (Figures 1 and 2) did not occur for the numerous small glands (sensilla hastiformia = spear-organs [2]) that do not release any secretion when the tick is pressure stimulated or when the legs are pinched [2, 3] (data not shown). Thus, secretory release and resultant changes in gland morphology apply to the large wax glands only.

3.3. Time to Replenish Large Wax Glands. Table 1 shows initial mass of different stages and mass change data as a result of having secreted. Adult females lose 2.3% of their mass after secretory reserves have been exhausted. The amount of the secretion in males accounts for approximately the same percent difference (2.5%) as that observed in females (ANOVA; $P > .05$). Nymphs lose 1.4% of their body mass after secretion and larvae lose 1.3% of their body mass

(ANOVA; $P > .05$) that was about one-half the amount observed in the adult stages (ANOVA; $P < .05$). No secretory activity was noted in freshly killed specimens at any stage (freeze/thaw or HCN) and corresponds to a lack of detection of any measurable mass change after their legs are repeatedly pinched with forceps (Table 1). Upon restimulation (until the amount lost remained constant), full capacity to secrete was regained after 12 days for adult females, 10 days for adult males, 6 days for nymphs, and 8 days for larvae.

4. Discussion

The structure of the large wax gland in adult *R. sanguineus* shows nearly the exact microscopic anatomy as original illustrations of the *sensillum sagittiforme* figured by Schulze [2] and is the same in all stages of the life cycle as described by Dinnik and Zumpt [1]. This glandular organ during their discussions is labeled as a secretory gland. At no time during development are the large wax glands morphologically redundant, embryonic, or nonfunctional [1]. Indeed, all stages have large wax glands, all stages secrete from these structures when they are disturbed, and all stages are protected against predation by ants [3, 6] that links the morphological occurrence of these large wax glands to semiochemical functioning. The sensory function of large wax glands that Dinnik and Zumpt [1] propose is entirely correct; most likely, it is of proprioceptive nature because of the mechanical force that triggers release of secretion by these glands and the identification histologically of axial fibers of nerve cells. Thus, the sensory function of the large wax

TABLE 1: Number of days required for the large wax glands to replenish secretion by different nonfed stages of *R. sanguineus* (93% RH, 25°C) based on restimulation (leg pinching) following initial exhaustion of secretory reserves. L, larva; N, nymph; AM, adult male; AF adult female; dead tick, killed by freezing and then thawed to room temperature (shown); *, results from HCN-killed were similar. Data are mean $\pm$ SE and values followed by the same superscript letter within a column are not significantly different (ANOVA; $P < .05$). (N = 3 replicates of 10 ticks for each time point with each replicate coming from a separate rearing batch of ticks).

| | % body mass lost after exhausting secretion | | | |
	L	N	AM	AF
Stimulated once				
Dead tick*	0	0	0	0
Live tick	1.25 ± 0.12^a	1.39 ± 0.24^a	2.51 ± 0.09^a	2.33 ± 0.17^a
Restimulation (day)				
0	0^b	0^b	0^b	0^b
2	0.24 ± 0.13^c	0.77 ± 0.09^c	0.39 ± 0.11^c	0.54 ± 0.18^c
4	0.71 ± 0.07^d	1.02 ± 0.23^d	1.24 ± 0.08^d	0.92 ± 0.13^d
6	0.82 ± 0.20^e	1.41 ± 0.14^a	1.16 ± 0.07^e	1.73 ± 0.16^e
8	1.21 ± 0.08^a	1.32 ± 0.20^a	2.10 ± 0.12^f	1.67 ± 0.06^f
10	1.30 ± 0.09^a	1.48 ± 0.12^a	2.44 ± 0.07^a	2.03 ± 0.19^g
12	1.22 ± 0.17^a	1.41 ± 0.15^a	2.63 ± 0.09^a	2.29 ± 0.24^a
14	1.36 ± 0.21^a	1.44 ± 0.09^a	2.58 ± 0.17^a	2.26 ± 0.11^a
Body size				
Initial mass (mg)	0.0347 ± 0.003	0.147 ± 0.009	2.16 ± 0.54	3.32 ± 0.26

gland is to perceive changes by stretching, pressure (cuticular deformation), or shifts in the chitinous exoskeleton that activates the secretory response. Schulze [2] alludes to such a mode of activation of the sensilla sagittiformia (large wax glands) as well.

From this study it is clear that the large wax gland is a holocrine pressure-secreting gland; therefore, the tick allomonal defense secretion is a holocrine secretion. Until now classification of the large wax gland based on how it operates was not known. Our criteria for assigning a holocrine function to the large wax gland include (modified from Fawcett [26]): (1) there is no conduit for the secretory material to reach the cuticular surface because the scolopale projecting edges prevent communication between the terminal chamber proximally and the distal duct chamber, thus ruling out the possibility of a secretion that is merocrine or apocrine in origin; (2) essentially the entire organ is gutted from the inside out in response to physical stimulus to the cuticle; (3) entire intact cells, or large fragments of cells, are ejected from the gland onto the cuticular surface. The long chain hydrocarbons that are the active ingredients of the allomone and function to hide the tick from ants [12] are likely liberated from the cells (so-called derived epidermal cells) when they are ejected and burst onto the cuticular surface. Release of internal contents when the cells burst probably serves an additional purpose by providing a sticky, viscous-type of consistency to the exudates, and this is also a characteristic of certain allomones that function uniquely against ants. The presumed function of such a viscous secretion is to facilitate clogging of the ant's antennal chemosensory receptors, along with blocking by the hydrocarbons, so that the tick goes undetected or is invisible to the ants chemoreception (modified from Whitman et al. [10]).

Based on morphology and collected data from this study we speculate the following mechanism for how this gland may function. In response to pressure stimulating the tick's legs, or entire body, by pinching or pressing with forceps, the physical forces produced from the forceps likely pull or stretch the exoskeleton that cause bending of the tuft within the tuft chamber. This stimulates the axial fiber of the sensory neurons that is directly linked to the tuft through the filament and knot of the scolopale. The nerve action potential produced from the bending of the tuft is sent to adjacent neurons causing the tick to respond by extending its legs out behaviorally followed by an immediate retraction of the legs under the body and remaining motionless for several minutes as though dead [3]. Conceivably, all of this muscle movement produces a burst of increased hydrostatic pressure that forces the glandular cells up into the distal terminal chamber and also causes wave-like rebound movements of the spongy elastic chitin surrounding the gland. As the cells are forced up into the terminal chamber they widen the chamber width and push the spongy compressible boundary material against adjacent cuticle that is more stable and stretch resistant. This forces the spongy elastic material to move in one direction, up toward the surface of the integument. The whole process of hydrostatic forces and the movement of the spongy boundary material facilitate the inside of the gland to widen and push the glandular cells up toward the surface. The large glandular cells' forceful ejection destroys the floor of the scolopale and tuft and forces the glandular cell debris into the lumen of the distal arrow duct and out the pore onto the surface of the cuticle.

Microscopic analysis of the large wax gland after having secreted also provides insight into how the gland renews and has the ability to secrete again. Like the majority of defense secretions it is delivered frugally [7, 10], but necessitates the replenishment of the proximally located glandular cells and associated structures because the secretion is holocrine based on the present data. All glandular cells are typically epithelial in origin that includes the hypodermal cell layer under the cuticle [26, 27]. Microscopic examination reveals that the glandular cells transdifferentiate from the squamous epithelial cells that make up hypodermis. This transdifferentiation includes a change in shape of the cells adjacent to the gland from squamous to cuboidal and a change in cytoplasmic morphology from an ungranulated to heavily granulated cytostol. This is also suggested from the observations of Schulze [2] where these cells are called regenerative cells. The nearly complete destruction internally of the large wax gland caused by forceful secretion of the basal glandular cells indicates that the glands would be nonfunctional for a long period of time as they are rebuilt, requiring many days (this study [3]). The complete exhaustion of all large wax glands on a tick, however, is unlikely to occur in a natural setting. Delivery of the defense secretion in slight pulses [3] and aimed at the site of attack (thus, not all tick's large wax glands secrete at any one time) requires that such cellular restoration occur, only intermittently, and this is consistent with the frugal delivery that characterizes allomonal defense secretions [10] so as not to be rendered totally defenseless. Also, as evidenced in unstimulated ticks (not secreted), the cells adjacent to intact glands have already started the differentiation process; thus, if a gland is exhausted it recovers more quickly because the regeneration process has already been started or even completed before the gland secretes.

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Research Article

Extremely Long-Closed Galls of a Social Aphid

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The aphid *Nipponaphis monzeni* (Hormaphidinae, Nipponaphidini) forms large, hard, completely closed galls on the evergreen *Distylium racemosum*, its primary host, in south-western Japan. By marking 100 galls on a tree and monitoring them over five years, and by sampling many immature galls from another tree in various seasons and dissecting them, we found that galls of *N. monzeni* are initiated in June, that they remain small for at least 21–22 months and that tiny fundatrices survive for over one year. Some galls rapidly expand during April/May in the third year. Others remain small and swell up in the fourth year and still others in the fifth year. Full-grown galls open in November/December, and alates fly to evergreen oaks, the secondary host. Thus galls of *N. monzeni* take 2.5 years to mature at earliest (3-year life cycle) and some galls 3.5 or 4.5 years (4- or 5-year life cycle).

1. Introduction

In general, aphid galls formed on a tree wither and die within the year [1], but a few exceptions have been reported from the subfamily Hormaphidinae. It takes longer than one year for galls of *Tuberaphis styraci* (Cerataphidini) to mature on the temperate deciduous *Styrax obassia* [2]. Galls of *Ceratoglyphina styracicola* (Cerataphidini) grow slowly and last for up to 20 months on the subtropical evergreen *Styrax suberifolius* [3]. *Nipponaphis distyliicola* (Nipponaphidini), on the evergreen *Distylium racemosum*, forms completely closed galls that remain small over several months and rapidly expand in the following spring [4]. Although not yet confirmed, galls of some other cerataphidines may also last for over one year [5–7]. These galls are (or are supposed to be) biennial and do not last beyond two years. We found extremely long-lasting galls in the Nipponaphidini, which remain closed for at least two and a half years.

Species of the tribe Nipponaphidini induce galls on *Distylium* trees (Hamamelidaceae) in eastern Asia [8–10]. The tribe includes several social species that produce defensive nymphs in the galls [11–15]. About a dozen nipponaphidines are known to form galls on *Distylium racemosum* in Japan [16, 17]. Among them, *Nipponaphis monzeni* forms the

largest and hardest galls on the tree [18, 19]. The mature brown galls (Figure 1(d)) are up to 8.5 cm in height [19] and the gall wall becomes lignified and so hard that one cannot crack them with bare hands. Even adult Japanese monkeys (*Macaca fuscata yakui*) are not always successful in opening the galls with their teeth [20]. Because of the conspicuous size and hardness, galls of *N. monzeni* are well known among people living in south-western regions of Japan where trees of *D. racemosum* are commonly planted. The old empty gall is called "Saru Bué (monkey whistle)" [21] and children blow into it through the exit hole to whistle [21, 22]. This species is also peculiar in that its first-instar nymphs repair their gall in a self-sacrificing manner, by discharging a large amount of body fluid [23, 24]. It has been unknown, however, when and how galls of *N. monzeni* are initiated and developed to mature. Through our preliminary (unpublished) study with a few galls, we have noticed that galls of *N. monzeni* last beyond two years. Later, by marking many galls, we confirmed that it takes more than two years for the galls to mature and open, and that there are three groups of galls that mature in different years; galls of one group grow to mature in the third year, galls of another group in the fourth year, and galls of the last group in the fifth year. This peculiar process of gall growth is herein reported.
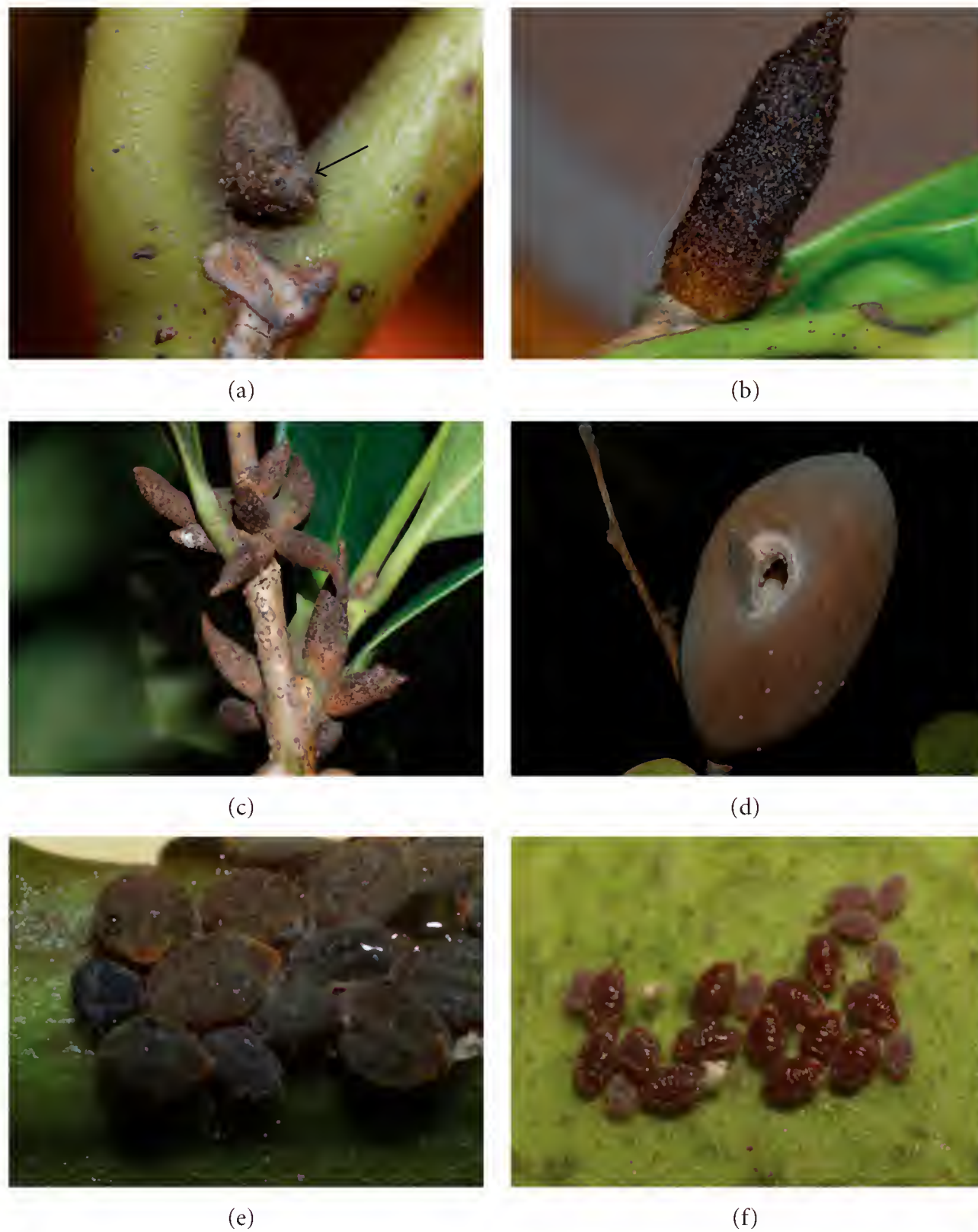
FIGURE 1: *Nipponaphis monzeni*: (a) an overwintered tiny gall (on 12 March 2008) that was formed on the base of an axillary bud of *Distylium racemosum* in the previous year; (b) after the first winter such a gall has grown to a cone-shaped gall (on 19 May 2008); (c) several cone-shaped galls with the hardened wall (on 29 October 2007); (d) a full-grown gall with a round exit hole (on 8 December 2008); (e) secondary-host generation (apterae and nymphs) on a twig of *Lithocarpus edulis* (on 9 January 2008); (e) sexuals on the underside of a leaf of *D. racemosum* (on 30 April 2008).

2. Hitherto Reported Life Cycle of *Nipponaphis monzeni*

The aphid *Nipponaphis monzeni* migrates obligatorily between *Distylium racemosum*, its primary host, and ever-green oaks such as *Quercus glauca, Q. myrsinaefolia,* and *Castanopsis sieboldii*, its secondary hosts, and induces galls on *D. racemosum* in south-western regions of Japan [18, 19, 23]. The mature galls are ellipsoid or fig shaped, about 35–86 mm and 26–66 mm in the major and minor diameters, respectively [19]. The gall wall becomes lignified, very hard, and up to 3.4–3.7 mm thick [18, 19]. In the end, late in autumn, a round opening appears (just as a submarine hatch opens; see Figure 1(d)) and one gall may produce 600–880 alates [19]. The alates (emigrants) migrate to leaves of oaks and give birth to first-instar nymphs on the underside. These

first-instar nymphs settle on twigs and become scale-like apterous adults [18] (Figure 1(e)). Nymphs produced by the apterous adults all develop into alate sexuparae in the following spring. No aphids remain on the secondary host during summer [8, 18]. The alate sexuparae fly back to leaves of *Distylium* and produce males and sexual females there [18] (Figure 1(f)). They copulate and the females deposit eggs onto the bases of buds [25]. First-instar fundatrices, which induce galls, hatch from these eggs in May [8]. Because galls of *N. monzeni* rapidly become large and hence noticeable during May, it has been supposed that eggs hatch soon and that the fundatrices quickly develop their galls to the full size, almost within a month [18, 26]. However, small galls of *N. monzeni* were found already in March and even earlier, before the return migration of alates from oaks. This fact suggests that galls of *N. monzeni* might remain small over

a long period, as is shown for *N. distyliicola* [4]. The prime purpose of the present paper is to make it clear whether this is the case.

3. Materials and Methods

3.1. Study Tree and Inducing Galls. A tree (Tree N) of *Distylium racemosum*, which had been planted in our garden, Niiza (35.79°N), Saitama Prefecture, Japan, was used as a "host" tree. We introduced a total of 2026 alate sexuparae of *Nipponaphis monzeni* onto the tree between 12 and 29 April 2001. *Distylium racemosum* is not naturally distributed in Saitama Prefecture. Around the garden, there were no natural colonies of *N. monzeni* on oaks, nor were trees of *D. racemosum* during the experimental period (from 2000 to 2006). These sexuparae were collected from a colony on a tree of *Quercus glauca* in Atsugi, Kanagawa Prefecture, and from another colony on a tree of *Q. myrsinaefolia* in Niiza, both of which had been established by the introduction of many alates (emigrants) from 18 galls of *N. monzeni* to the oak trees. The 18 galls were collected from trees of *D. racemosum* in Shinkiba (about 29 km south-east of Niiza) along Tokyo Bay on 6 December 2000. The alate sexuparae were placed on the upper sides of leaves one by one with a pair of forceps. To prevent them from taking off, we wetted a target leaf with water before placing alates on it. These alates were stuck on the leaf but, after the water evaporated, most of them walked onto the underside and larviposited there.

3.2. Rearing of Sexuals. A cut-off twig of *Distylium racemosum* was placed in a plastic container, and 53 alates that had been collected from the two colonies on the oaks were introduced into the container between 23 and 26 April 2001. When eggs were found, they were kept in glass vials under room temperature to determine approximately when eggs hatched.

3.3. Monitoring Galls. New shoots began to grow on Tree N in late March or early April during the experimental years. We made a map of some branches of Tree N and recorded the position of incipient galls on the map. Between 24 September and 12 October 2001, 55 galls were mapped. At that time, because all galls were very tiny (about 1 mm or less in width), soft, and fragile, we did not measure the exact size. Between 28 April and 6 May 2002, additional 43 galls were found and mapped. On 24 November 2002 and on 3 June 2003, two more galls, which had been overlooked before, were marked. We recorded whether these 100 marked galls were alive (and opened finally) and measured the width and height with vernier calipers to the nearest 0.1 mm up to nine times: (1) between 28 April and 6 May 2002, (2) on 23 and 24 November 2002, (3) on 9 and 15 March 2003, (4) on 3 June 2003, (5) on 3 December 2003, (6) on 30 May 2004, (7) on 5 December 2004, (8) on 22 May 2005, and (9) on 14 December 2005. The sizes of measured galls are shown in the text as mean ± SD, together with range and the sample size (*n*) in parentheses. Because we carelessly overlooked a gall twice, this sample size does not exactly accord with the number of live galls.

3.4. Sampling of Immature Galls. To know how a colony of *Nipponaphis monzeni* develops within the gall, young galls were sampled from trees in Shinkiba in various months. Because zero- to 15-month-old galls could certainly be distinguished from older galls, these age-knowable galls were selectively sampled from a tree (Tree S1), which harbored many old and live galls of *N. monzeni,* from October 2007 to September 2008: on 29 October, 9 January, 12 March, 4 April, 16 April, 30 April, 19 May, 2 June, 16 June, 7 July, 5 August, and 17 September. From nine to 29 age-knowable galls were collected on each day. A few additional galls were sampled from other trees there. We also sampled many older (age-uncertain) galls from Tree S1 on 31 March 2007 and 2 June 2008. They were deposited in 80% ethanol and later were measured and dissected to examine the colony structure. Since galls of *N. monzeni* were completely closed, dead aphids remained in the galls. The number of live and dead aphids were counted. The age of the sampled galls is estimated based on the assumption that they were formed in June and is indicated in months. In 2008, new shoots began to grow on Tree S1 between 16 and 30 April. On several other trees in Shinkiba buds had already burst by 16 April.

3.5. Identification of Galls. Because cone-shaped galls (Figures 1(b), 1(c)) of *Nipponaphis monzeni* are peculiar in structure and appearance, it was easy to distinguish them from immature galls of other nipponaphidines on *Distylium racemosum* at the time of sampling. Tiny semispherical galls (Figure 1(a)) of *N. monzeni* much resemble those of *N. distyliicola* and *Monzenia globuli*, both of which are formed on (mainly axillary) buds and were seen on trees in Shinkiba. Galls of *M. globuli* are initiated about one month earlier (in May), and offspring of the fundatrix appear already in July [27], while galls of *N. monzeni* are initiated in June and the fundatrix does not produce offspring until the next spring (see Results). Hence there is little possibility of misidentification between the two species. On the other hand, galls of *N. distyliicola* are initiated about one month later (in July) and the fundatrix produces offspring from October onward [4]. There would therefore be some possibility of misidentification for semispherical galls sampled in August and September. However, galls of *N. distyliicola* were much fewer than those of *N. monzeni* in Shinkiba during 2006–2008, particularly on Tree S1, from which we sampled most galls of *N. monzeni*; hence, misidentification, if any, would hardly affect our results.

3.6. Examination of Morphology of Aphids. Many aphids in young galls were macerated in 10% KOH solution, stained with acid fuchsin or Evans blue, and mounted in balsam (for method, see, e.g., [28]), and their morphology was examined under a light microscope.

4. Results

4.1. Reared Sexuals and Eggs. The alate sexuparae placed on leaves (and those introduced into the plastic container) soon
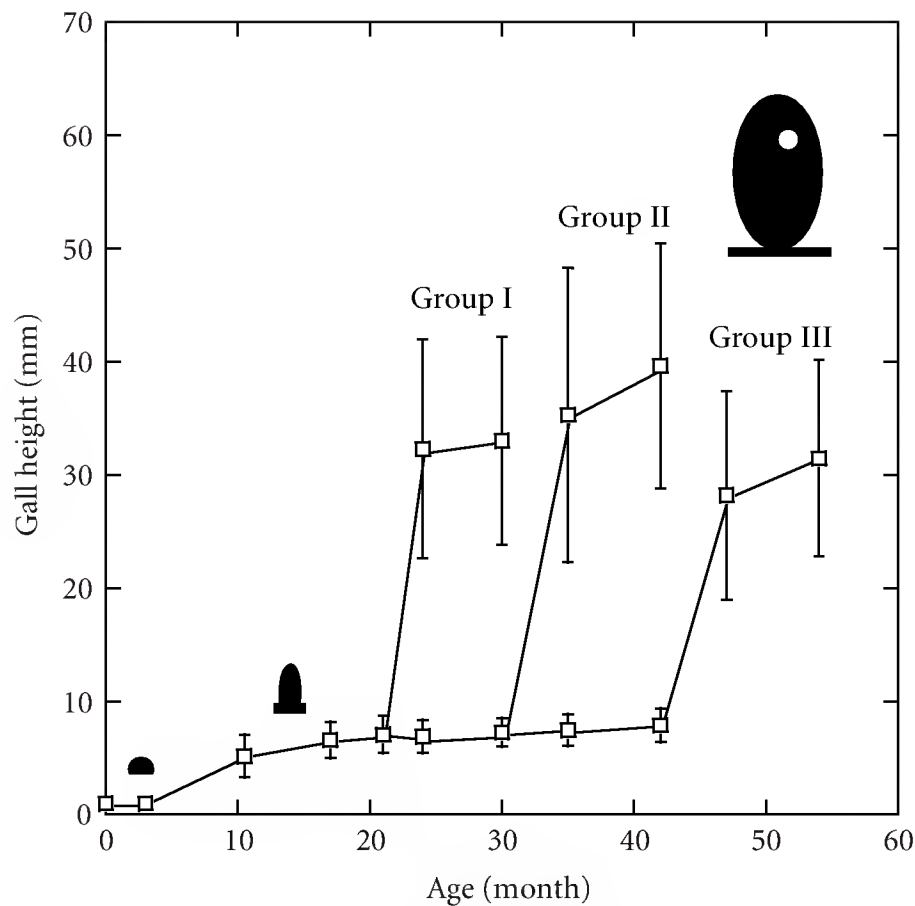
FIGURE 2: Development of galls of *Nipponaphis monzeni* formed on a tree (Tree N). Gall height is indicated as mean (square) ± SD (vertical bar). Groups I, II, and III correspond to the first, second, and third expanding groups, respectively, in the paper.

gave birth to sexuals that were reddish brown in color on the underside. On 11 May 2001 we noticed eggs laid in the container for the first time, on the bases of leaf blades, sides of midribs, and near the margin of curled leaves. On 14 May 54 eggs found in the container were transferred into glass vials. Between 23 and 30 May 2001 six eggs hatched into first-instar fundatrices, but the others died without hatching. This accords with Takahashi's [8] remark that, in Osaka, eggs of *Nipponaphis monzeni* hatch in May.

4.2. Development of Monitored Galls.
The development of the monitored galls on Tree N for up to 54 months (4.5 years) is summarized in Figure 2.

4.2.1. Fundatrices and Incipient Galls.
On 13 June 2001 many first-instar fundatrices were found walking on twigs of Tree N. Incipient galls were already formed on axillary buds of leaves. Many of them were not yet closed up. We found many closed small galls in September 2001. They were semispherical, about 1 mm or less in diameter, tinged with brown and covered with white hairs.

4.2.2. Galls after 10 or 11 Months.
Between 28 April and 6 May 2002, approximately 10 or 11 months after the gall formation, 32 (58.2%) of the 55 incipient galls marked the last year were found alive. The live 32 galls and 43 newly marked galls were slender in shape, 2.5 ± 0.7 mm (range 1.0–4.6) in width and 5.2 ±1.9 mm (range 1.2–9.7) in height ($n = 74$).

4.2.3. Galls after 17 Months.
On 23 and 24 November 2002, approximately 17 months after the gall formation, 74 of the 75 marked galls were found alive. An additional gall, which had been overlooked, was newly marked. All these galls became slightly larger but still were cone shaped. They were 3.2 ± 0.6 mm (range 2.1–5.0) in width and 6.6 ±1.6 mm (range 3.5–10.8) in height ($n = 75$).

4.2.4. Galls after 21 Months.
On 9 and 15 March 2003, 71 of the 75 galls were found alive. These galls were still small, and 3.2 ± 0.6 mm (range 2.0–5.0) in width and 7.1 ± 1.6 mm (range 3.6–11.2) in height ($n = 71$). Hereafter, these galls developed to mature, but in three different years, that is, in the third (2003), fourth (2004), or fifth (2005) year.

4.2.5. Galls after 24 Months.
On 3 June 2003, 68 of the 71 galls were alive. Of the 68 galls, 40 (59%) had begun rapidly growing and became swollen and were 28.1 ± 6.6 mm (range 16.9–41.4) in width and 32.3 ± 9.6 mm (range 19.0–61.3) in height ($n = 40$). Those galls that grew to mature in the third year are referred to as galls of the first expanding group. On the other hand, the remaining 28 (41%) were still small. One hitherto overlooked cone-shaped gall, which did not expand in this year, was newly marked. Those that did not swell up were 3.5 ± 0.8 mm (range 2.4–5.4) in width and 6.9 ± 1.4 mm (range 3.4–8.9) in height ($n = 29$).

4.2.6. Galls after 30 Months.
On 3 December 2003, all (29) galls that had not expanded in this year remained closed and alive and were 3.5 ± 0.7 mm (range 2.4–5.0) in width and 7.3 ± 1.3 mm (range 4.0–9.2) in height ($n = 29$). Of the 40 galls of the first expanding group, two failed and 32 were open. Of the remaining six, five successfully opened by 31 December 2003, but one did not open and failed. The 40 galls, including the failed three, were 28.3 ± 6.4 mm (range 17.2–40.9) in width and 33.0 ± 9.1 mm (range 19.8–60.9) in height ($n = 40$).

4.2.7. Galls after 35 Months.
On 30 May 2004, of the 29 galls that had not swelled up in the third year, nine (31%) were expanding, of which one was broken, perhaps by a bird. These galls, referred to as of the second expanding group, were 36.3 ± 9.3 mm (range 21.9–51.0) in width and 35.3 ± 13.0 mm (range 20.0–56.8) in height ($n = 9$). The other 20 (69%) remained small and were 3.9 ± 0.8 mm (range 3.0–5.6) in width and 7.5 ± 1.4 mm (range 5.0–10.1) in height ($n = 20$).

4.2.8. Galls after 42 Months.
On 5 December 2004, of the nine galls of the second expanding group, the broken one was found dead, and the remaining eight were open and were 40.5 ± 9.0 mm (range 22.7–52.7) in width and 39.6 ± 10.8 mm (range 29.9–57.0) in height ($n = 8$). The 20 galls that had not swollen in the fourth year, still remained small and were 4.2 ± 0.8 mm (range 3.2–5.6) in width and 7.9 ± 1.5 mm (range 5.5–11.1) in height ($n = 20$).

4.2.9. Galls after 47 Months. On 22 May 2005, all 20 galls were expanding. These galls (referred to as of the third expanding group) were 34.0 ± 10.9 mm (range 12.8–47.7) in width and 28.2 ± 9.2 mm (range 14.0–46.5) in height ($n = 19$). Thus, of the 69 galls that were alive on 3 June 2003, 40 (58%), 9 (13%), and 20 (29%) turned out to be of the first, second, and third expanding groups, respectively.

4.2.10. Galls after 54 Months. On 14 December 2005, of the 20 galls of the third (last) expanding group, one was found dead and 15 were open and the remaining four were still closed. The live 19 galls were 38.6 ± 7.6 mm (range 18.1–50.0) in width and 31.5 ± 8.6 mm (range 17.3–46.4) in height ($n = 19$). The fate of the four galls had been followed until 8 October 2006. One of the four withered and fell off the tree, and the three contained many dead aphids including alates when cracked on 8 October. Once-swollen galls that had not opened by January did not open thereafter.

Of the 15 successfully opened galls, four were marked between 24 September and 12 October 2001, which no doubt indicates that some galls last for over four years.

4.2.11. Galls Formed on a Single Bud. More than one gall was at times formed on a single bud. Of 65 buds on which our mapped galls were formed, 20 harbored more than one gall (up to five). On eleven of the 20 buds, more than one gall (up to four) successfully developed and later expanded to mature. On seven of the eleven buds, all galls (up to three) expanded in the same year (on five buds in 2003, on one in 2004, and on the remaining one in 2005), while on the other four buds at least one gall expanded in a different year.

Also on other trees in the field (in Shinkiba), both cone-shaped and expanded galls were at times found at the position of a single bud.

4.3. Sampled Galls and Colony Development. Colony development for the first 15 months is summarized in Figure 3.

4.3.1. Incipient Galls. In Shinkiba many sexuparae and sexuals of *Nipponaphis monzeni* were found on the undersides of leaves of *Distylium racemosum* on 30 April 2008 (Figure 1(f)). Many tiny newly-formed galls of *N. monzeni*, some of which had not yet closed up, were found on buds of Tree S1 on 16 June 2008. These galls each contained a first-instar fundatrix. Some fundatrices were just attacking buds. Galls were formed on axillary buds of newly developed shoots or on auxiliary buds of terminal buds, or rarely on terminal buds themselves.

Such tiny yet completely closed galls (Figure 1(a)) were found from July to March/April. These galls each contained a single fundatrix. The fundatrices were still first instar in August, and eight out of 21 fundatrices were 2nd instar on 17 September. They were probably adults from late October onward. Tiny galls collected on 8 April still contained a single fundatrix only, but one gall already contained a first-instar nymph of the second generation.

4.3.2. From 10- to 15-Month-Old Galls. Tiny galls on buds began to develop from April/May in Shinkiba (Figure 1(b)).
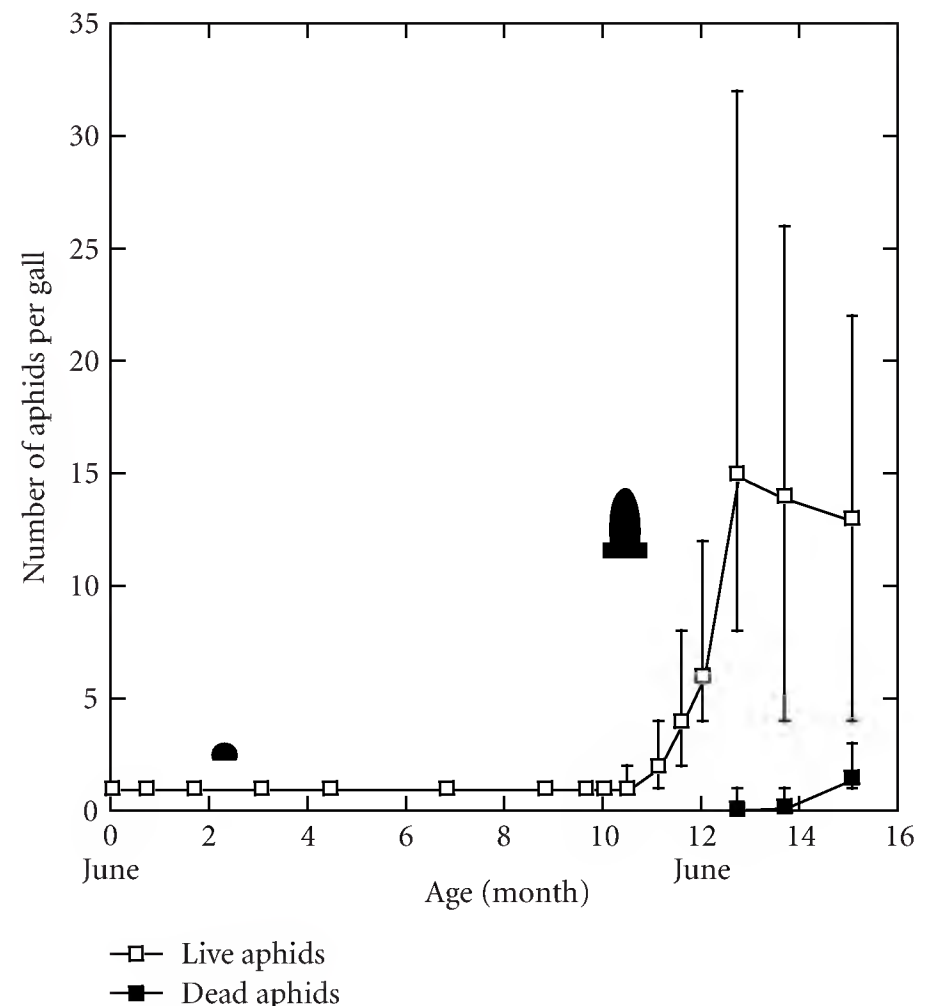

FIGURE 3: Colony development in galls of *Nipponaphis monzeni* for the first 15 months. Vertical lines indicate the range for the number of aphids per gall. The sample sizes are 20, 17, 20, 21, 13, 20, 15, 17, 23, 25, 17, 29, 20, 13, 9, and 20 from the left to right. The ages are calculated based on the assumption that all galls were initiated on 15 June.

Most of them were still 1 mm or less in height on Tree S1 during April, and up to 1.7 mm on 19 May. (We found one gall which was 7.9 mm in height on another tree in Shinkiba on 30 April 2008.) These galls contained a live fundatrix and 0–3 nymphs of the second generation. The galls grew further, reaching 5.5–11.2 mm in height on 7 July. In many cases, the axillary bud on which a gall had been formed sprouted; then, the gall was located at the base of a new shoot. In others, the axillary bud did not sprout; the bud might later be atrophied. During this period, colony size also increased, reaching 8–33 on 7 July (Figure 3). In most galls, the fundatrix was still alive after one year. Dead fundatrices were found in one out of 13 (13-month-old) galls on 7 July and two out of nine (14-month-old) galls on 5 August. Thus, fundatrices of *N. monzeni*, despite their small size, live for over one year (Figure 3). There were usually two (sometimes one or three) apterous adults with thickened hind legs in addition to several nymphs. By September the gall wall became thick and hard, and galls ceased growing. From 20 (15-month-old) galls collected on 17 September, no live fundatrix was found. Eight of the 20 galls contained one dead thick-legged aptera or two besides the dead fundatrix (one of the galls also contained one dead nymph).

4.3.3. Galls Collected at the Beginning of June. One hundred cone-shaped galls collected on 2 June 2008 were dissected. They were 2.5 ± 0.7 mm (range 0.6–5.1) in width and 5.8 ± 1.8 mm (range 1.0–10.1) in height. These galls would include

those that passed one, two, and three wintering seasons, or 12-, 24-, and 36-month-old galls. (Besides them finally expanding galls were seen on the tree. They would be 24, 36, or 48 months old.) There was in fact only a weak correlation between gall surface area (calculated by assuming that the gall is a right circular cone) and the number of live aphids ($r = 0.65$) or the total number of live and dead aphids ($r = 0.62$). (With gall volume instead of surface area, the correlation coefficients decreased to 0.59 and 0.56, resp.; cf. [29].) The number of live aphids in the galls is shown as a histogram, together with information on the number of dead aphids, in Figure 4(a). It was easy to discriminate 12-month-old galls from 24- and 36-month-old galls because the former contained no dead aphids, while the latter two contained at least one dead aphid that was well mummified. In addition, the gall walls of the former were still soft and the surfaces were tinged with red (Figure 1(b)), while those of the latter two were hard and the surfaces were dark brown with dust (for some exceptional cases, see Section 4.4). Twenty-nine of the 100 galls were 12 months old (Figure 4(a), white area on the lowest bar). On the other hand, it was not easy to discriminate between 24- and 36-month-old galls. The number of dead aphids varied from one to 37 (mean 4.5, exclusive of those containing no dead aphid). The galls with one or a few dead aphids were likely to be 24 months old, and two that contained more than 20 (24 and 37) dead aphids were likely to be 36 months old.

4.3.4. Galls Collected at the End of March.
One hundred cone-shaped galls collected on 31 March 2007 were dissected. They were 2.9 ± 0.7 mm (range 1.4–5.4) in width and 6.6 ± 2.2 mm (range 2.2–12.0) in height. These galls had not yet resumed growing at that time and the gall walls were hard. The galls would include those that passed two, three, and four wintering seasons, or 21-, 33-, and 45-month-old galls. The number of live aphids in the galls is shown as a histogram in Figure 4(b). The number of dead aphids varied from one to 150 (mean 9.9) and six galls contained more than 50 dead aphids. We could not accurately determine which galls were 21, 33, or 45 months old, but the six galls were likely to be 45 months old and many of those that contained only a few dead aphids to be 21 months old. The number of live aphids ranged from eight to 202 (mean 67.0), and 57 of them contained more than 50 aphids. There was only a weak correlation between gall surface area and the number of live aphids ($r = 0.69$) or the total number of live and dead aphids ($r = 0.63$).

It is worth mentioning here that a fair quantity of wax but little honeydew remained in these galls.

4.4. Some Exceptional Cases.
Galls of *Nipponaphis monzeni* formed in June remain very small until the next spring and grow to cone-shaped galls from April to June in the second year (Section 4.3). However, some galls that had not fully grown in this period were obtained (Table 1). These galls (e.g., two galls collected on 29 October in Table 1) were still very small after one year and contained only two or three live aphids besides one or two dead aphids. During growing
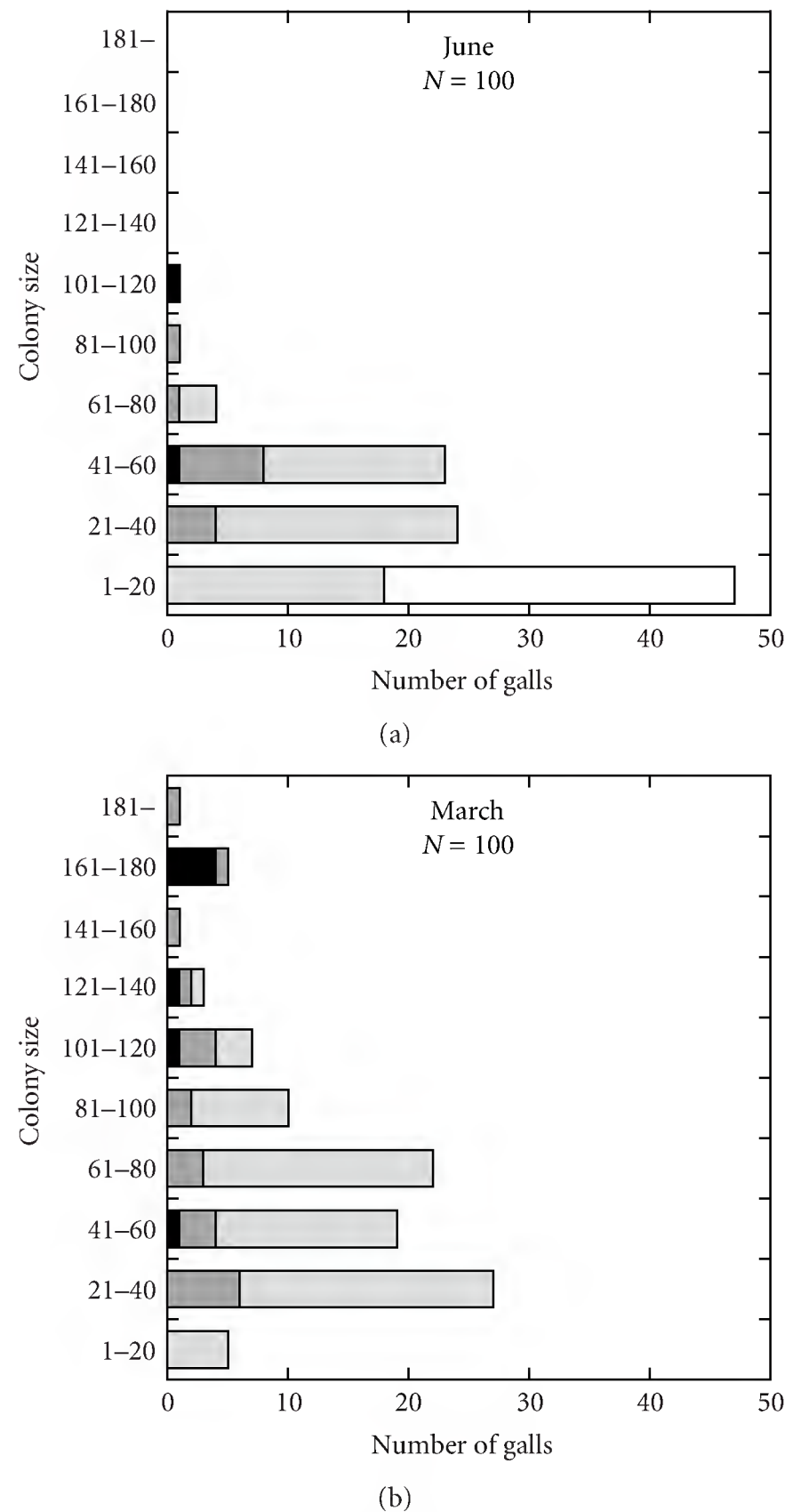

(a)

(b)

FIGURE 4: Colony size (number of live aphids) for 100 cone-shaped galls of *Nipponaphis monzeni* collected on 2 June 2008 (a) and 31 March 2007 (b). Those galls that contained zero, from one to five, from six to 20, and more than 20 dead aphids are indicated by white, light gray, dark gray, and black areas, respectively, on the bars.

season (e.g., on 2 June 2008), several newly grown cone-shaped galls, which contained from one to four dead aphids, were found (Table 1). The dead aphids were well mummified, indicating that they had died long before then. It is very likely that such galls did not grow in the second year and became cone shaped in the third year. These cone-shaped galls would expand to mature in the fourth or fifth year (i.e., after three or four wintering seasons).

4.5. Morphology of Aphids in Young Galls.
The adult fundatrix of *Nipponaphis monzeni* was very small, with short, three-segmented antennae and no cornicles (Figure 5(a)). Mounted specimens were only 0.37–0.47 mm long (mean

TABLE 1: Some galls of *Nipponaphis monzeni* whose growth seems to have been delayed.

Gall[a]	Date of collection	Height and width in mm	No. of live aphids	No. of dead aphids	Estimated age in month
07102-17	29 Oct. 2007	? × 1.2	2	2	16
07106-8	29 Oct. 2007	2.4 × 1.9	3	1	16
08054-4	2 June 2008	7.6 × 3.4	16	2	24
08054-10	2 June 2008	1.8 × 1.4	3	2	24
08054-21	2 June 2008	4.7 × 2.0	8	2	24
08054-22	2 June 2008	4.9 × 1.9	7	2	24
08054-23	2 June 2008	3.1 × 1.6	6	1	24
08054-31	2 June 2008	4.4 × 2.1	7	1	24
08054-41	2 June 2008	6.9 × 2.7	26	4	24

[a]All galls were collected from Tree S1 in Shinkiba, Tokyo.
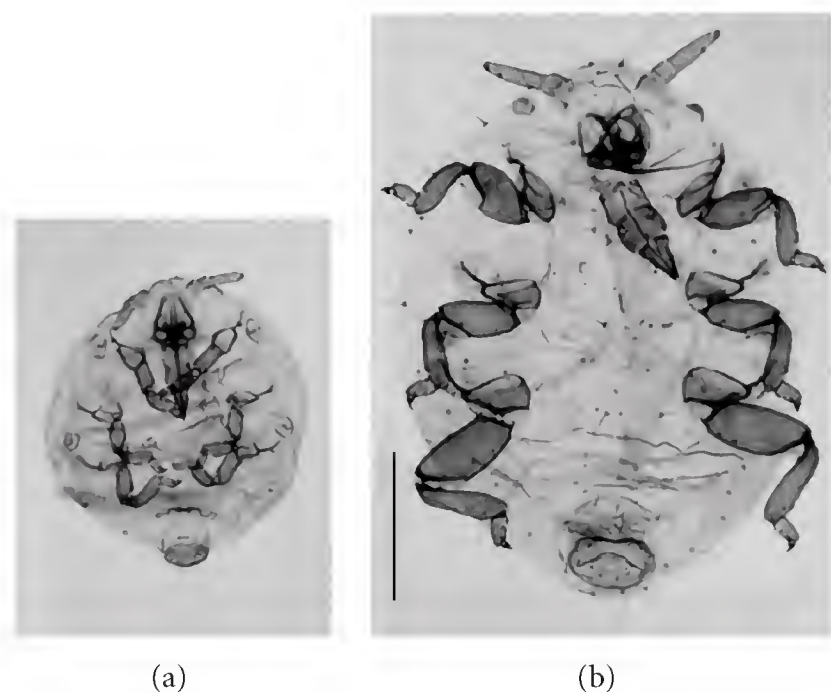

FIGURE 5: Adults of *Nipponaphis monzeni* in young galls: fundatrix (a) and thick-legged aptera (second generation) (b). Note the difference in size between them (shown at the same magnification). Scale bar: 0.2 mm.

0.42, $n = 10$). As mentioned before, one or two offspring of the fundatrix develop into apterous adults with greatly thickened hind legs (Figure 5(b)) in May/June of the second year, or in 11- or 12-month-old galls. These thick-legged apterae were much larger than the fundatrix; mounted specimens were 0.60–0.76 mm long (mean 0.68, $n = 10$), usually (but not always) with a pair of small cornicles. Many thick-legged apterae were also found in those galls that were finally expanding during this season (but they were larger than those produced in cone-shaped galls). By dissecting some expanding galls, we observed that these thick-legged apterae were repeatedly scratching the inner wall of the gall with their thickened hind legs (our unpublished observations recorded on video). Dead thick-legged apterae were found from September onward in cone-shaped 1-year-old galls. From September, when galls had ceased growing, apterous adults with normal (i.e., not thick) hind legs appeared instead of thick-legged apterae. This suggests that the scratching by thick-legged apterae might play some role in the process of gall growing or hardening. Some thick-legged apterae had developed embryos.

4.6. *New Records of Secondary Hosts.* As the secondary hosts of *Nipponaphis monzeni*, *Quercus glauca* [18, 19], *Q. myrsinaefolia* [23], and *Castanopsis sieboldii* [19] have been reported. In Shinkiba, colonies of *N. monzeni* were commonly found on *Quercus phillyraeoides* and *Lithocarpus edulis* (Figure 1(e)) as well as on *Q. glauca* during winter. As other researchers reported [8, 18], no colonies of *N. monzeni* remained on these secondary hosts during summer.

5. Discussion

5.1. *Life Cycle.* The present study has made it clear that galls of *Nipponaphis monzeni* initiated in June remain small over a long period. They pass winter at least twice and some of them three times or even four times as small cone-shaped galls (as tiny semispherical galls during the first winter). It takes almost 2.5, 3.5, or 4.5 years for galls of *N. monzeni* to produce alates in December (Figure 2). Although we have focused on galls on a single tree or two (Trees N and S1), the same pattern of delayed gall development was observed on many other trees in Shinkiba and Meguro, Tokyo. From June to December these trees harbored at least three types of galls: tiny semispherical galls, small cone-shaped galls, and expanded large galls. In May/June, new and old cone-shaped galls, and expanding large galls were seen on these trees. In addition, both cone-shaped and expanded galls were at times found at the position of a single axillary bud (Section 4.2.11). These facts strongly suggest that our finding on Tree N is a rule and not an exception. Sorin [18] and Okuno et al. [26] mentioned that the life cycle of *N. monzeni* is annual. This is clearly not the case. Their mistake is understandable because, when their articles were published, no aphid galls that last for more than one year had been known.

As mentioned before, alates that have crawled out of the gall fly to evergreen oaks and their progeny form open colonies on the twigs. Unlike many other nipponaphidines (e.g., *Nipponaphis distyliicola* [4, 18], *Metanipponaphis cuspidatae* [8, 30, 31], *M. rotunda* [32], *Quadrartus yoshinomiyai* [17]), no aphids remain on the secondary host after alate sexuparae fly back to *Distylium racemosum* in April/May. Thus, it takes three, four, or five years for *N. monzeni* to complete its life cycle. In this system, gene flow is assured between yearly cohorts.

5.2. Origin of the Peculiar Life Cycle. Although the life cycle of *Nipponaphis monzeni* may seem strange, similar yet shorter life cycles have been reported in two other nipponaphidines. Fundatrices of both *Monzenia globuli* [27] and *Nipponaphis distyliicola* [4] form tiny incipient galls on buds of *Distylium racemosum* in Japan. Galls of *M. globuli* are initiated in May and remain small during the first three or four months and rapidly swell up in September [27]. Galls of *Nipponaphis distyliicola* are initiated in July and remain small until March or April and grow to the full size in May [4]. Galls of the former species are annual and those of the latter biennial. It is likely that ancestral galls of *N. monzeni* remained small for a shorter period as do present galls of *M. globuli* or *N. distyliicola*.

5.3. Why Such a Long Life Cycle? It is rather surprising that a colony of sap-sucking insects can persist for such a long period (for up to 4.5 years) in the completely closed gall. The fundatrix of *Nipponaphis monzeni* survives for over one year (Figure 3), and other individuals may also survive over several months, judging from the number of dead aphids remaining in the galls (Figure 4). This raises the question how the aphids dispose of their honeydew, which would be dangerous to their life [33, 34]. The aphids produce wax, but wax-coated globules of honeydew, which are commonly seen in galls of both social and nonsocial aphids [35–37], were not detected from their galls. It remains unknown whether they excrete little honeydew or they remove honeydew in some way.

It is also unknown why some galls of *N. monzeni* take 2.5 years to mature, while others 3.5 or 4.5 years. Although aphids inside the galls perhaps may not enter "diapause" in its physiological sense, this phenomenon is akin to prolonged (or delayed) diapauses, which are known in various insect groups [38] such as weevils [39–41], gall midges [42], and carnid flies [43], and often are interpreted as a bet-hedging adaptation [39]. In the case of *N. monzeni*, it is also possible that gall inhabitants may avoid competition for nutrition within a yearly cohort that has formed galls on the same or nearby buds. Large galls might draw assimilates more widely from source areas on the plant than small galls, as has been shown for fordine aphids on *Pistacia* [44–46].

Despite these unanswered questions, there is one clear advantage of their long-lasting galls. During the final expanding phase of the gall (in April/May), nymphs of *N. monzeni* repair their still-soft gall in a self-sacrificing manner when a hole is bored through the wall [23, 24]. This tactics can work well only if there are many "repairers," or first-instar nymphs, in the gall. In fact, galls that were expanding in April/May contained approximately 700–2400 live aphids [23]. For aphid species that form annual galls, as each begins with a single fundatrix, it will be difficult to attain such a large colony size at this time. Even in the congener *N. distyliicola*, whose galls are biennial, the colony size during the final expanding phase is approximately from 20 (in late April) to 190 (in late May) [4]. Because of the long pre-expansion period, many galls of *N. monzeni* contain from 50 to more than one hundred live aphids already in March (Figure 4(b)).

This permits the colony to quickly attain a large colony size and hence to prepare many repairers.

Acknowledgments

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Research Article

Trigona corvina: An Ecological Study Based on Unusual Nest Structure and Pollen Analysis

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We found that the nest of *Trigona corvina* (Apidae; Meliponini) consists mainly of pollen exines from bee excrement, forming a scutellum shield encasing the colony. A 20-year-old nest (1980–2000) from a lowland Panama forested habitat was sawed in half longitudinally, and a 95 cm transect was systematically sampled each 5 cm. Samples subjected to detailed pollen analysis held 72 botanical species belonging to 65 genera in 41 families. Over 90% of scutellum pollen volume was Cecropiaceae and Arecaceae, among $> 10^{13}$ grains. Potentially the oldest samples, in the middle of the nest, indicate that Mimosoideae, Euphorbiaceae, and Bombacaceae (now Malvaceae) were lost when Africanized honey bee competitors colonized Panama in 1984. *Cecropia* deposited in the nest increased markedly after landscape-level vegetation disturbance. Pollen from *Cavanillesia* demonstrated that the foraging range encompassed 3 km^2 and perhaps 500 plant species. *Trigona corvina* primarily foraged on plants with large inflorescences, consistent with foraging theory considering their aggressive behavior.

1. Introduction

Relatively few stingless bees, tribe Meliponini, build their entire domicile, because most use pre-existing cavities [1]. Even fewer provide a record of plant resources they have consumed [2]. *Trigona corvina* does both. This Neotropical genus of over 40 species [3] consists primarily of aggressive bees living in nests exposed on small branches or large surfaces—not within tree or ground cavities [3–5]. A 140 kg nest of *Trigona corvina* broke from its supporting tree-top branch after 20 years, and DWR happened to observe both inception of the nest and its fall. It was rolled on logs into the back of a pickup truck and taken to a cold storage room at the Smithsonian Tropical Research Institute, Tupper Building, in Balboa, Panama. The nest was split longitudinally with a chain saw. Half was shipped to the Hunterian Museum in Glasgow, Scotland, now on permanent display as an exemplar of animal architecture. The remainder is described and analyzed here. We applied melittopalynology—the analysis of bee-collected pollen [6].

2. Methods

Foundation of the colony was noted at the site by DWR in 1980, during a visit to R. L. Dressler. In Curundu Flats, Ancon, a large patch of several thousand worker *T. corvina* was observed at 15 m from the ground on an ornamental tree, *Lagerstroemia* (Lythraceae) at the Dressler residence. The nest was completed during approximately one month as an ovoid dark mass [5, 7] around a 10 cm diameter apical stem of the tree. The size of the nest steadily increased during the next 20 years, and it was observed frequently. The mature nest had an outer covering with irregular holes of approximately 1–3 cm diameter, separated by 1–3 cm from each other, and long, slender tubercles of approximately three to ten cm in length surrounding the lower half of the ovoid nest entrance tube, which projected a few centimeters from the nest surface (Figure 1). On the day the nest fell it was retrieved and placed in cold storage, with defensive bees still inside, as stated in Section 1.

Later the nest was sawed in half from top to bottom. A transect was made along the maximum length and a cubic cm

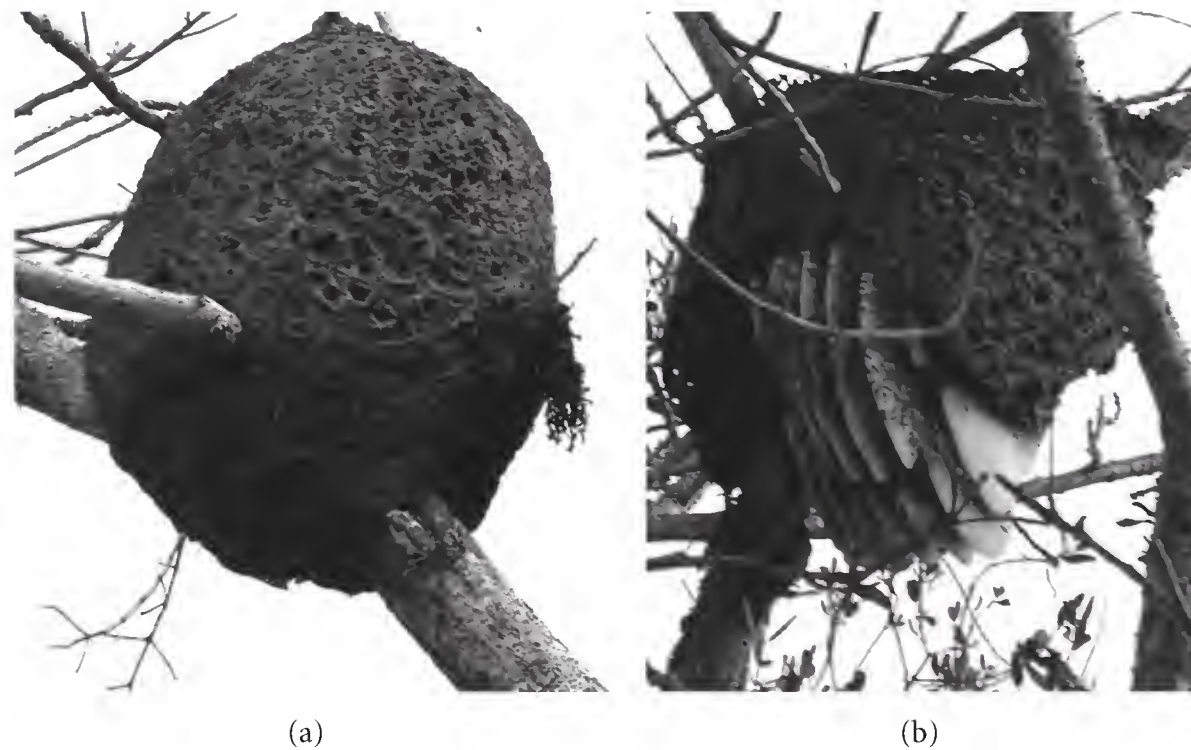(a)   (b)

FIGURE 1: (a) Active nest of *Trigona corvina*, Cerro Campana, Panama. (b) Active nest of Africanized *Apis mellifera* established in the scutellum of *Trigona corvina* near Summit Gardens, Panama city, Panama.
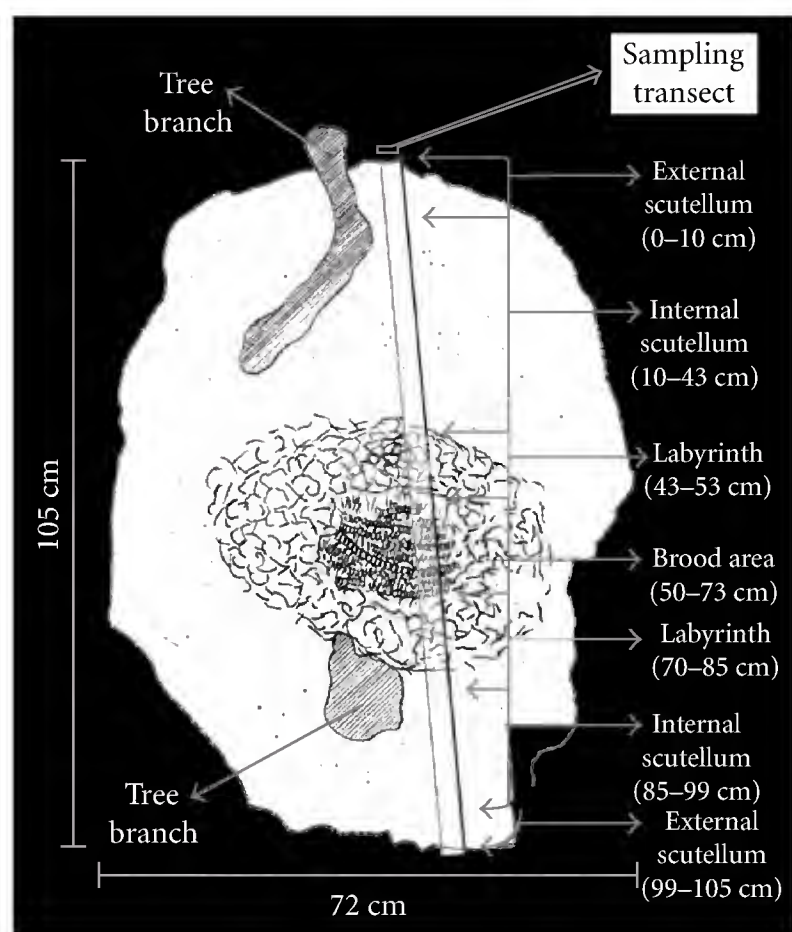

FIGURE 2: Diagram of scutellum and entire nest of *Trigona corvina* and the sampling transect.

of material was removed at 30 points, every 5 cm (Figure 2). Eleven of the samples, spread evenly along the transect, were chemically treated using standard laboratory pollen "acetolysis" protocols with concentrated acids to destroy organic debris, clear, and slightly stain pollen grains [8]. Two tablets of the fern marker spore *Lycopodium* were added to each sample as a reference standard [9]. This technique allows pollen grain counts to be compared on a volumetric basis, where each *Lycopodium clavatum* (batch 938934) tablet provided roughly 11 000 spores. The proportion of pollen grains to spores indicated their number per cubic cm. Pollen identifications of the acetolyzed pollen were based

on collections and a pollen atlas for central Panama [10]. Photomicrographs were made of each species. A pollen diagram, developed for stratigraphic studies, used Tilia and Tilia.graph [11]. For comparative purposes, a single honey sample from a colony of Africanized *Apis mellifera* and one of *Tetragonisca angustula* (Meliponini) were analyzed, using 50 mL of honey (see [12]) taken from the pooled honey contents of the nest, at a Cuurundu site near the former Dressler residence, in 2002.

3. Results

The scutellum (the enclosure made by the bees and surrounding their nest) was thick and hard, covering the entire nest, particularly the upper portion (Figure 2). Seventy-two species and 65 genera in 41 plant families were identified in the total nest scutellum transect (Figures 3 and 4). Samples of the 15 most voluminous pollen species in the diet of *Trigona corvina* are depicted in Figure 3. The five palm species were not only best represented as a single family but had high total pollen percentages within the cubic cm samples— from 30% to 50%. *Roystonia* (an introduced species) was dominant, and *Cocos nucifera*, also an introduced species, was present in relatively large amounts. The nectarless secondary growth tree *Cecropia peltata* attained counts of 23% to 47% of total grains in individual samples from the transect. Most remaining species of 39 families were insignificant to sporadic (Figure 4), but more noteworthy were Bombacaceae (now called Malvaceae, 4 species), Burseraceae (1 species), Euphorbiaceae (4 species), and Anacardiaceae, all of which are trees. The dominant herb pollen was *Mimosa pudica*, a nectarless scandent shrub. Lianas had low representation, including *Machaerium* (Papilionoideae) and the nectarless *Davilla* (Dilleniaceae). *Tetragonisca angustula* (Figure 5) among the commonest nesting meliponines in the area did not use resources closely similar to either *Apis* or *T. corvina*.
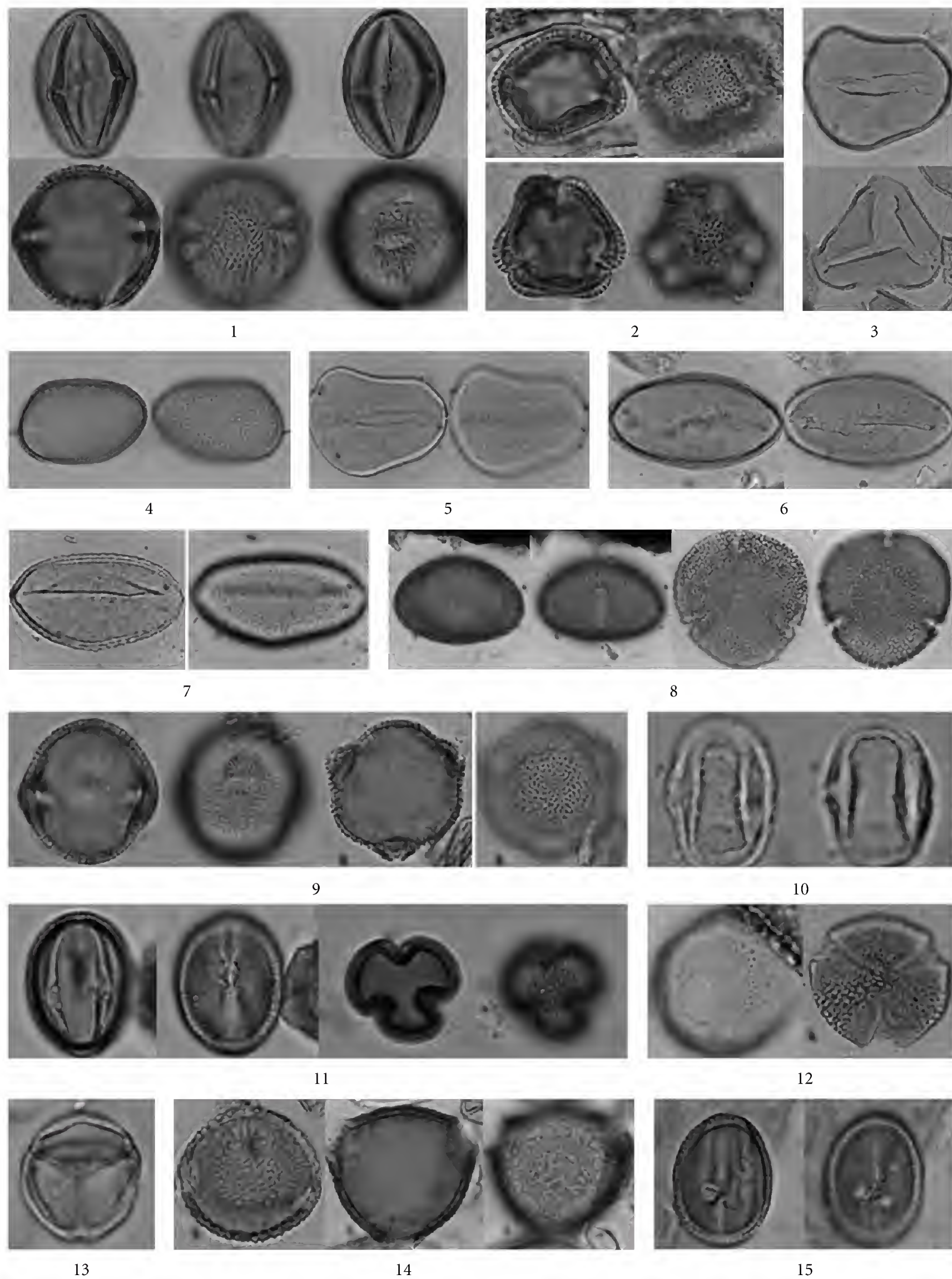
FIGURE 3: Representative common pollen species taken from nest scutellum. Anacardiaceae: *Spondias mombin* (1); Araliaceae: *Didymopanax morototoni* (2); Arecaceae: *Cocos nucifera* (3); *Crysophilla warcewiczii* (4); *Elaeis oleifera* (5); *Phytelephas* (6); *Attalea butyracea* (7); Bombacaceae: *Pseudobombax septenatum* (8); Burseraceae: *Bursera simarouba* (9); Cecropiaceae: *Cecropia peltata* (10); Euphorbiaceae: *Chamaesyce* (11); Dilleniaceae: *Davilla nitida* (12); Mimosoideae: *Mimosa pudica* (13); Papilionoideae: *Erythrina fusca* (14); *Machaerium* (15). Images not to scale, photographs ×1000.

FIGURE 4: Scutellum pollen stratigraph of the 20-year-old nest of *Trigona corvina*.

FIGURE 5: Pollen graph for a honey sample analyzed from the study area in mid 2002, from a resident colony of Africanized honey bees and from an abundant native stingless bee, *Tetragonisca angustula*.

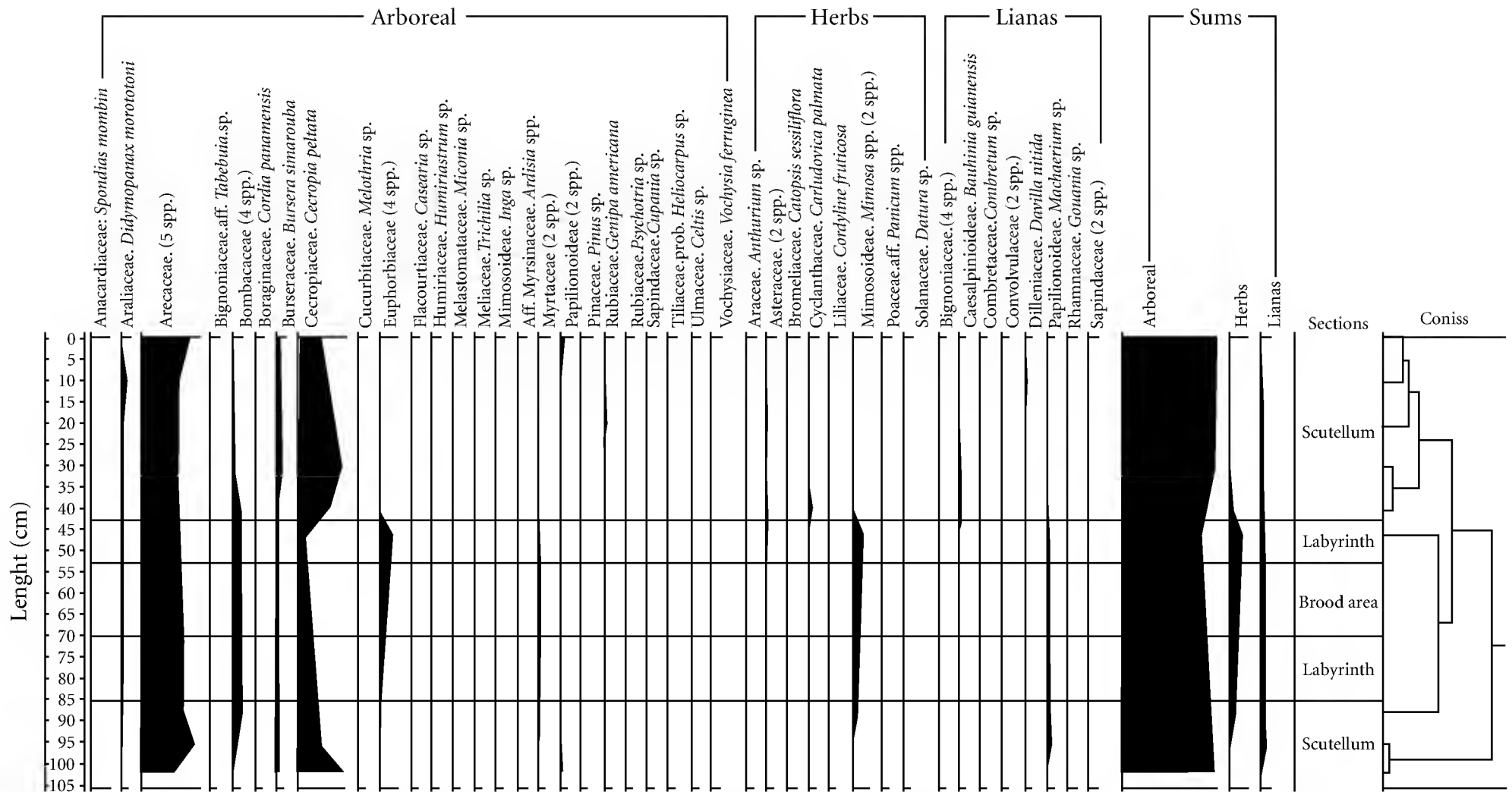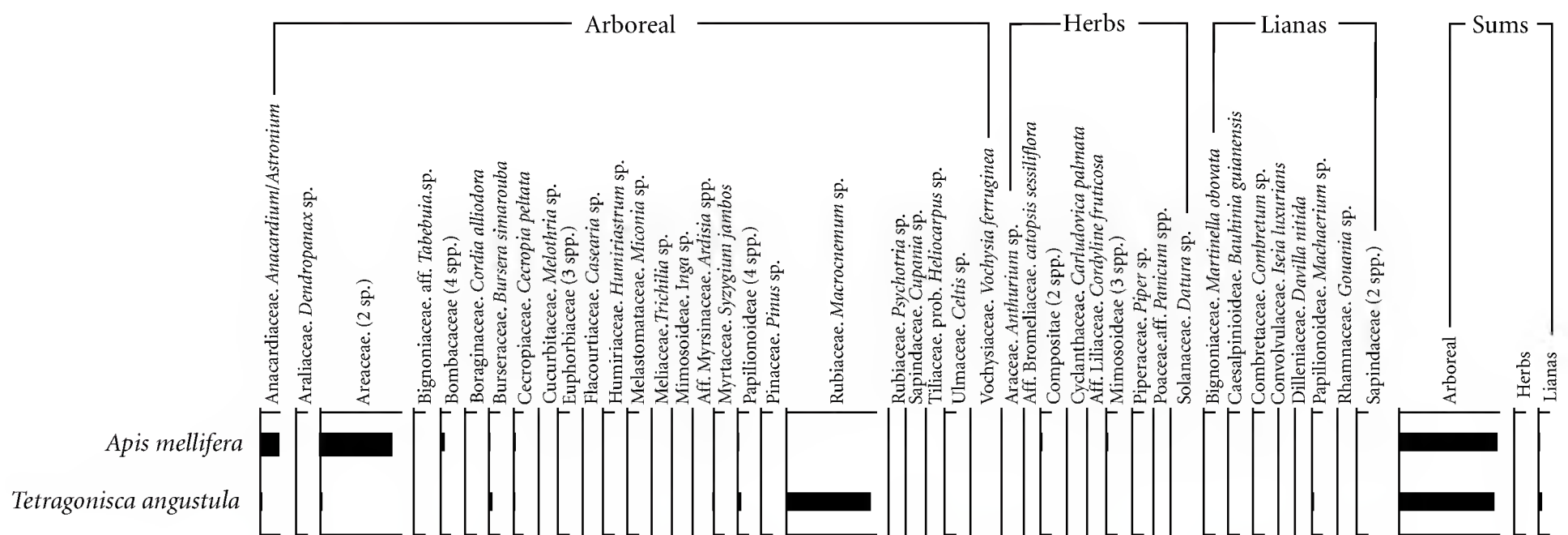The pollen stratigraphic diagram given in Figure 4 indicates a process of pollen deposition by bees in the nest scutellum, and the volumentric contribution of each species and family, over time. The larger extent of scutellum above the brood area is seen in Figure 2. On the far right of the graph, the "CONISS" is a cluster analysis of similarity in nest composition among transect samples. The composition pattern of pollen deposition by the brood area and labyrinth (the nonbrood or food area within the scutellar covering of the nest) were generally similar, as were the samples taken

within the upper or lower scutellum. These form the three main branches of the dendrogram. The upper scutellum and mid nest were of similar composition, while the lower scutellum was the outlier—least similar in grain species and abundance.

The pollen grain concentration per cubic cm was extraordinary, from 27 to 49 million. If an egg-shaped nest structure is modeled on this basis [13], then the nest, an egg-shaped object measuring 105×72 cm maximum diameter, was made from the pollen or exines of 2.5×10^{13}

(25 trillion) grains. This estimate does not subtract volume of the labyrinth, involucrum, brood and stored honey, and pollen, in the center of the nest.

4. Discussion

Nest Biology. The pollen study yielded a wealth of data not only on bee biology but also on vegetation dynamics. Early studies of nest architecture in Neotropical *Trigona* mentioned an earthen, clay, or cerumen and resin scutellum, incorporating vegetation and also vertebrate feces. These were found not to be major scutellum components. The scutellum is reminiscent of decaying wood. Nogueira-Neto [4] first clarified that the scutellum of *T. spinipes*, under microscopic examination, consisted primarily of the normal debris collected within the nest—fecal meconia and pupal exuviae, plus some mites, cerumen, resin (sometimes called geopropolis), and disarticulated bees. He did not mention pollen, but the meconium is pollen voided in the cell before the cocoon is spun by a mature larva. It is a thin layer on the base of the cocoon. Further work with garbage pellets ejected outside the nest of an Asian stingless bee [14] verified that bases of used brood cells and pollen exines were the major garbage components. The accumulated solid debris in the nest cavity of four nests of *Cephalotrigona zexmeniae* was analyzed and found to consist of identifiable pollen, and little else [2].

After foundation of the nest and its initial growth, the brood area of *T. corvina* was encased in an increasingly thick layer of excreted pollen taken from the emerged brood cells and ostensibly deposited first in refuse piles within the nest, as noted for stingless bees in general [1, 15]. We speculate that workers did not eject pollen feces or garbage pellets in the normal way but incorporated them into the external nest area, outside the involucrum, and gradually formed the thick scutellum. Young adult bees may also contribute directly to this deposit, by defecating on the scutellum after pollen-rich initial adult meals (see also [4]). The farthest outer regions of the nest are sheathed in thin concentric sheaths of hardened and brittle resin or cerumen, which crumble when touched, and allow defending bees to exit *en mass* [1]. Immediately inside the outermost sheaths and their supporting pillars, the internal scutellum was 43 cm thick above and 35 cm below. It is likely that no material initially filled such passageways. This area was gradually packed with the pollen feces, which solidified in the layers between what may have been sheaths of cerumen and resin, or the loosely arranged small connectives which formed a labyrinth (neither involucrum or food storage structures).

A young nest is approximately half the size of the old nest we examined [5] but the total brood area may undergo little change in the life of the colony. This may be the result of initial scutellum building. The middle 42 cm of the nest at the time it fell to the ground was occupied by the brood combs, pollen and honey storage pots, and a labyrinth of involucral sheaths and pillars that enclosed the area (Figure 2). Perhaps part of this area was the first to be protected by the pollen exine scutellum, and later, the

used brood cell bases with pollen feces and little or no other material were transferred toward the exterior of the nest. The brood area could thus not expand. The pollen taxonomic composition similarities indicated by the CONISS analysis and dendrogram (Figure 4), and similarities in the width of pollen deposition of *Cecropia* at the 95–100 cm area and the 0–40 level, suggest that pollen feces first filled space around the brood and on the top of the nest and later were deposited below the brood area. The properties of pollen exines, like an insect cuticle, are near indestructibility from sun, wind, rain, and ultraviolet radiation [16, 17]. Insulation from direct sunlight, commonly reaching 40°C at the exposed nest surface (DWR unpublished data, Panama Metropolitan Park, Smithsonian Canopy Crane), augments the nesting value of scutellum. In addition, defense by thousands of flying, aggressive workers protects the entire nest. Potential mechanical protection or support, from above or below, differed by about 10 cm of scutellum. We suggest that the scutellum provides defense yet little structural support, but insulates and protects the nesting colony. It is also possible that the small, basal scutellum seen in the nests of *T. dallatorreana* and *T. nigerrima* [3] is a result of young nest age, but it may also show that bees select nesting branches unable to support the weight of a full scutellum.

Only one meliponine genus, the Neotropical *Cephalotrigona*, does not eject its colony trash and instead accumulates a deposit of up to several kilograms in the base of the nest cavity. This compact bee excreta has been used to characterize the entire pollen spectrum of colony resources [2]. *Trigona corvina*, *T. spinipes*, and *T. amalthea* [4, 7, 18, 19] each make a full-size scutellum. Its texture, size, smell, and, in the first two cases, microscopic content indicate pollen exines and bee excreta. *Trigona amalthea* (syn. *T. trinidadensis*, nec. *T. silvestriana*; J. S. Moure, quoted on [18, page 135]) is the largest stingless bee of Mexico and Central America, but few nests have been dissected [7, 19]. One that was examined by the authors had a complete scutellum that was heavy. Several clades within *Trigona* [3] may also prove to have pollen records residing in excreta that comprise their nest scutellum. Unlike some indications [3] most of the nest scutellum in *T. corvina* was built on top of the nest (Figure 2). If bees do not switch colony behavior to perform normal meliponine trash ejection, the mature nest scutellum means that time is running out for the colony. For an exposed nest on a tree branch, or for a gigantic nest such as that of *T. amazonensis* on a tree trunk [3, 20], the weight of the nest may cause it to fall from the substrate. In kind, the accumulation of colony feces at the base of the nesting cavity of *Cephalotrigona* will eventually prevent colony survival.

Landscape Changes. The data indicate some interesting changes in pollen use by *T. corvina* which coincide with arrival of a major competitor, invasive Africanized honey bees, and with disturbances to the surrounding forest. Our interpretation of stratigraphic results from the bee nest includes landscape changes and colonization, in 1984, of Panama city and later western Panama by large numbers of Africanized honey bee colonies [21, 22]. The melissopalynological sample (honey [12]) from a nest of Africanized *Apis mellifera* also at Curundu Flats, and one of another resident

stingless bee, *Tetragonisca angustula*, less than 100 m from the nest site of *T. corvina* in Figure 5, are discussed below.

In our concept of nest construction, the central portion of the pollen diagram (Figure 4), consisting of transect points from approximately 40 to 85 cm, depicts the forage landscape when the colony was founded. In early 1982 the area surrounding the study nest was cleared of regrowth for nearly 400 m, as overgrown roads were cleared and repaved. This created an edge habitat in which *Cecropia*, a pioneer secondary growth tree, expanded along the newly opened areas (see [23, 24]). This pollen, among the dominant pollens used by *T. corvina*, was several times more abundant in the outer nest layers, particularly toward the top of the nest.

Honey Bee Impact and Stingless Bee Foraging Behavior. A second conspicuous change in the pollen profile is virtual disappearance of Euphorbiaceae, Bombacaceae (now Malvaceae), and Mimosoideae during a later time in the colony's existence. After the nest was four years old, colonization of central Panama by Africanized honey bees occurred [21], which use not only the three plant families mentioned above but also palms and *Cecropia* [24–26] (Figure 5). Recently, this invasive bee also was found using the scutellum of *T. corvina* to house a colony (Figure 1). In the colony pollen profile, *Trigona corvina* may have lost significant resources (see [27]) to the Africanized honey bees, while continuing to use pollen of palms, Burseraceae, and *Cecropia* (Figures 4 and 5). It is not known whether intensive competition led to the demise of the colony depicted in Figure 1(b), but it has been inferred that Africanized honey bees often concentrate on resources in more open habitats [28] and this is where nests of *T. corvina* also occur (pers. obs., [5, 29]).

The single honey samples of *A. mellifera* were collected in May of 2002 but had some of the same principal species noted for *T. corvina*. These are obviously not all nectar sources. All topical honey carries "contaminant" pollen from nectarless plants, such as *Cecropia*, *Mimosa pudica*, grasses, and *Davilla*. No quantitative comparison can be given of different plant species from pollen counts in honey, and thus no quantitative comparison can be made. For quantitative appraisal of which pollen sources are most important to Africanized honey bees, see [24, 25]. In the present discussion, relative importance of a plant species to *Apis* is not so relevant as the relative importance to its competitor (see [27]). *Apis mellifera* has a very large diet breadth but does specialize on highly rewarding flower patches, such as flowering trees [20, 25].

The "powerhouse" colonies of *Apis* and *Trigona* selected the richest local resources, but the unaggressively foraging *T. angustula* did not, as elucidated below. The degrees to which these species chose resources by species (rather than by their relative abundance), by competitor presence, or by density in the habitat are factors that must be considered in assessing whether any bee is "selective." For the moment, the best hypothesis seems to be that persistent, large, and dense inflorescences of common species, and therefore the best potential resources, were monopolized by *Apis* and *Trigona*. Palm inflorescences present a dense, highly rewarding pollen resource used extensively both by honey bees and highly aggressively group-foraging stingless bees. This pollen barely appeared in the honey of *Tetragonisca angustula* (Figure 5). The *Trigona corvina* is foraged by recruiting hovering groups of a few hundred foragers to tree canopies which are dominated by a few individual colonies [30]. The nests of aggressively foraging *Trigona* are not randomly distributed in the landscape but are regularly spaced [31] to avoid precipitous battles that occur between colonies attempting to dominate a rich, concentrated resource [20, 32]. *Cecropia peltata* also has clusters of catkin-like inflorescences with abundant pollen, often dispersed by wind.

The diet of *Trigona corvina* included *Cavanillesia* (Bombacaceae), and the source trees grow within Parque Metropolitano, at least 1 km from the nest. This observation allows an estimate of minimum forager activity range, and indicates colony exploitation of over 3.14 km^2. However, bees of similar size, *Trigona fulviventris*, forage at least 2 km from their nest, documented in a single instance on Barro Colorado Island, Panama, where a *Piper* inflorescence near the laboratory clearing received a visit from a bee marked at its nest on Zetek trail. Conservatively, the local flora within access to the colony of *T. corvina* contained some 500 species of flowering plants including 100 or more tree species [33], the main resources of *Trigona corvina*. Among these, *Trigona corvina* added exotic or disturbed forest-edge species to their principal plant resources. They used relatively few plants and growth forms, rarely using lianas or shrubs (Figure 4) [2].

Thus, similar to the "trace fossils" provided in coprolites (fossilized excrement) the scutellum in a nest of *T. corvina* provides a summary of the colony history and ecological factors that affect historical data. Rigorous analysis of pollen accumulations provides a new avenue for exploring and applying the information residing in stingless bee nests.

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Research Article

Nesting Behavior of *Abispa ephippium* (Fabricius) (Hymenoptera: Vespidae: Eumeninae): Extended Parental Care in an Australian Mason Wasp

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The genus *Abispa* includes Australia's largest wasps, potters with distinctive mud nests weighing up to 0.5 kg. During 31 days near Katherine, NT, Australia, we observed 8 active *A. ephippium* (Fabricius) nests and dissected 16. Nesting is lengthy and asynchronous; female generations often overlap. Females display long-term parental care through truncated progressive provisioning, removing debris, repairing damage, and attacking potential invaders. Males patrol water-gathering spots, and visit and associate with active nests, mating there and in flight. Females actively guard nests, but challenged nest-attending males simply retreat. The distinctive funnel-shaped entrance helps females defend nests physically but probably not chemically; dismantled for cell closure material, it is built anew for each cell. Nests contain up to 8 cells; construction and provisioning total about 7 days per cell. The only parasite was *Stilbum cyanurum* Forster. Thievery and nest usurpation by *Pseudabispa paragioides* (Meade-Waldo) were discovered.

1. Introduction

Restricted to Australia and New Guinea, the 6 described species of *Abispa* wasps are solitary-nesting mason or potter wasps that in the words of an early biologist, "take the palm for being one of the largest and handsomest" [1]. *Abispa* are in fact the largest Australian wasps, measuring up to 40 mm long, and their red/orange/yellow and black coloration is both striking and conspicuous. Their massive mud nests (Figure 1)—first described [2] in 1850—are equally outstanding, with a downward-pointing funnel-shaped entrance tube and unusually thick (up to 16 mm) mud walls. Nests commonly can weigh half a kilogram.

Knowledge of *Abispa* biology is sparse, limited to a few brief reports summarized elsewhere [3]. Although the wasps themselves are not uncommon in entomological collections, their nests are widely dispersed, well camouflaged, and often hidden in well-protected locations. The only long-term study [4] of nest construction behavior for any *Abispa* was based on observations of a single nest of *A. ephippium* (Fabricius);

it documented that females of this species were long lived (>2 months), and that nests were constructed slowly and were provisioned with caterpillars. There also has been only one report [5] of *Abispa* sexual behavior; it showed that males of *A. ephippium* and *A. splendida* (Guérin-Méneville) patrolled areas where females regularly collect water (an essential commodity in short supply in arid Australia) and perhaps at nests as well, and that males mated repeatedly at these sites with no evident courtship preliminaries.

Here, we report the first detailed behavioral study of *Abispa*. We provide new information on nest construction, provisioning, defense, usurpation, parasitism, and mating, with a unifying focus on the parental care displayed by females of *Abispa ephippium*.

2. Methods

2.1. Study Site. Nesting behavior was studied at the Northern Territory Rural College campus of Charles Darwin University, 15K N. of Katherine (S14^0 22.545′ E132^0 09.403′).

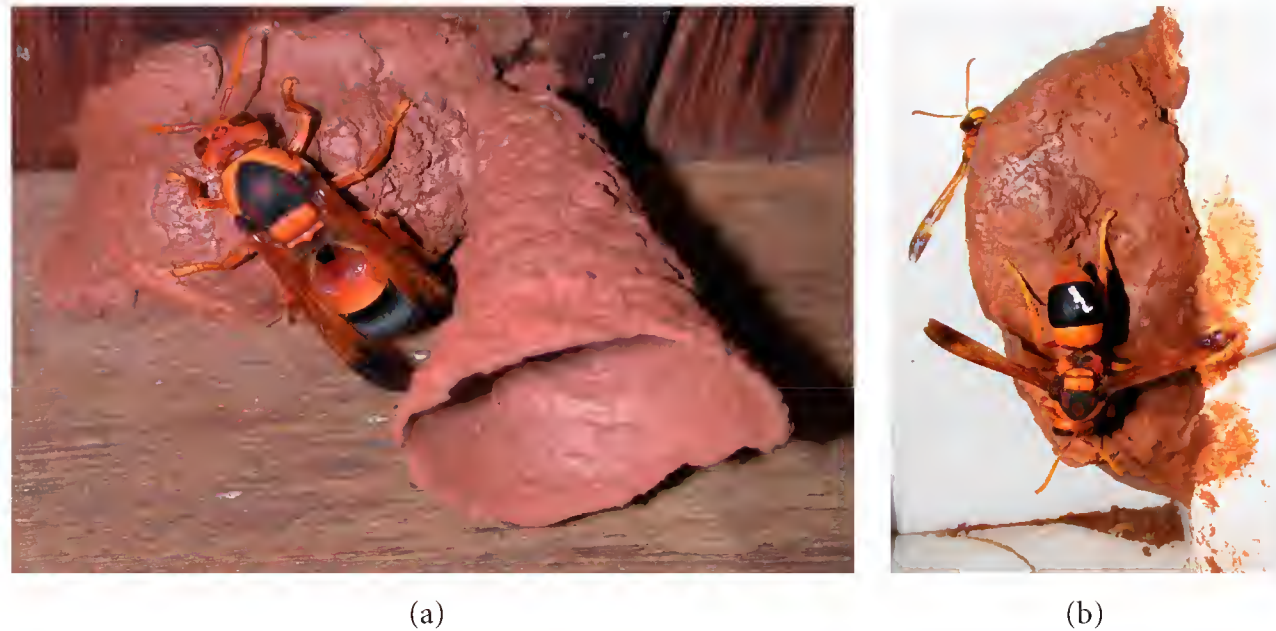(a)   (b)

FIGURE 1: Typical nests of *Abispa ephippium* Perkins from Katherine, NT, Australia. (a) A newly constructed single cell nest lacking the smooth exterior mud layer that characterizes older nests. (b) A mature nest with 7 cells (funnel out of view on back side) with a visiting male lurking at the back. The wasps measure about 35 mm long.

Nests were located on and beneath various campus buildings, always in rain-sheltered, low-light niches. Long-term observations were conducted principally under the drive-through carport and laundry area of a "Queenslander" style residence and around a small temporary pool nearby formed by a leaking hose fitting in the yard (Figure 2); in 2004 we simultaneously monitored nests on a daily basis at several nearby buildings.

2.2. Study Dates. Field studies were conducted from 27 November to 10 December 2004 and from 17 November to 5 December 2007, calendar intervals that correspond to a period of increasingly frequent rains that herald the rainy season in northern Australia. Heavy rains occurred intermittently, especially in the evening and at night, during both years. Daytime high temperatures generally ranged 90–95°F (31–35°C).

2.3. Behavioral Study Methods. To enable individual recognition, nesting female *A. ephippium* were netted and marked on the thorax, abdomen, or both with unique combinations of spots of white, green, and silver Liquid Paper correction fluid. Other individuals of both sexes flying through the area or visiting the small pool were opportunistically captured and also marked.

During each nesting season, a focal nest was chosen for detailed behavioral observations. The focal nest for 2004 was observed for a total of 58 hours spread over 14 days, during which 2 cell construction cycles were observed. The focal nest for 2007 was observed for a total of 63 hours over 11 days. In 2004, 4 additional nests were monitored daily; in 2007, 2 additional nests were collected after shorter observation. Digital photographs and video clips were obtained for documentation and more detailed analysis. At the conclusion of the field studies, all nests were collected and dissected.

2.4. Challenge Presentations. To roughly simulate encounters with natural parasites at different stages of construction or

(a)

(b)

FIGURE 2: Study sites. (a) Drive-through carport of a residence on the Charles Darwin University Rural Campus 15 km North of Katherine, NT, Australia, that was the main study site in both years. *Abispa ephippium* and other mason wasps nested among the carport rafters. (b) Small temporary pool under a mango tree in the residence yard; it was regularly visited by both *Abispa* sexes in 2004.

provisioning, resident females associated with 3 nests were challenged with freshly killed bombyliid flies and chrysidid wasps either affixed to the end of a long flexible palm-frond straw (Figure 3) or glued directly onto the nest exterior (Figure 4) using Elmers school glue. In addition, at other
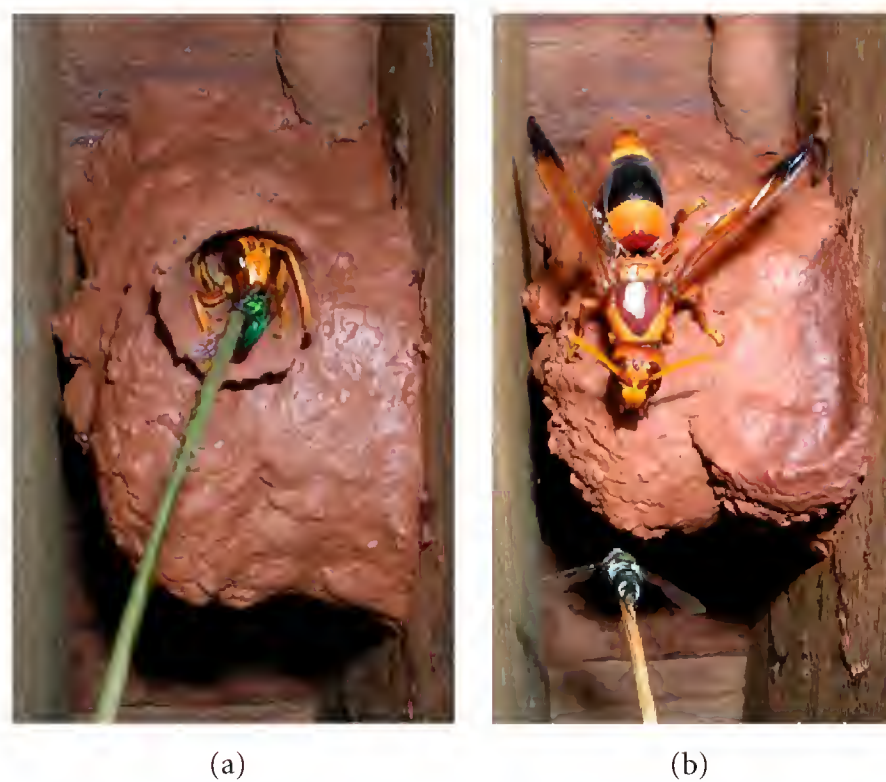
FIGURE 3: Intruder experiments. (a) A freshly killed cuckoo wasp (*Chrysis* sp.) and (b) a bombyliid fly (*Thraxan* sp.). When presented to a female *A. ephippium* at different stages in nest construction, both elicited a strong aggressive response.
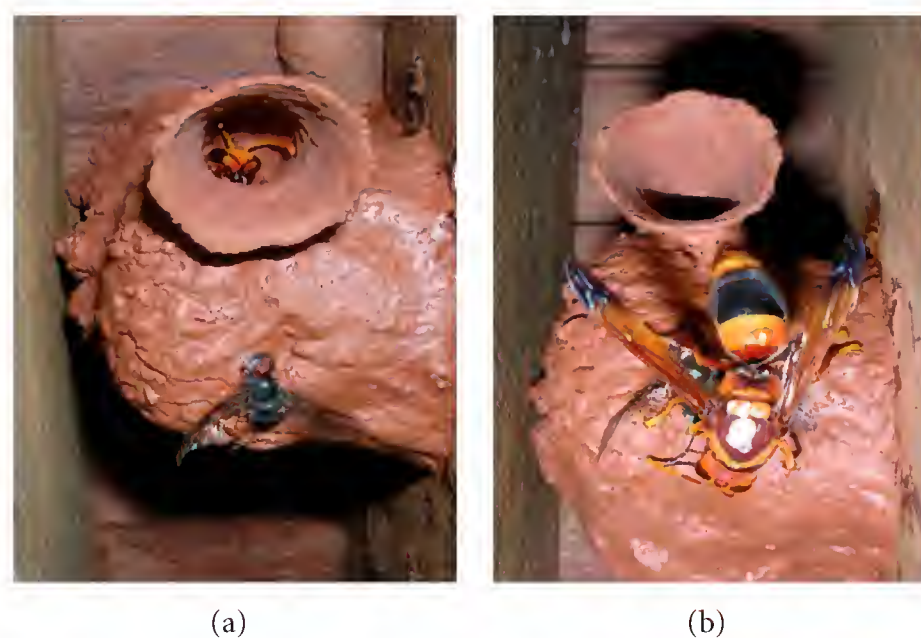

FIGURE 4: Experiment with a *Thraxan* "intruder" glued to nest surface (a). When the female encountered the fly, she attacked it viciously (b). Nest funnel diameter 15–17 mm.

times, we similarly presented either an insect-sized three-colored bead model or a freshly killed (by freezing) individual of either the social vespid *Ropalidia* or the gregariously nesting sphecid *Sceliphron formosum* (Smith), 2 species that commonly nested at the study site but were never observed to interact with *Abispa*. Because we had so few active nests, we did not intend these to be replicated, systematic, well-controlled experiments, but instead, preliminary "concept" trials.

Because the function of the funnel-shaped nest entry of *Abispa* and some other mason wasps is largely unknown, we also undertook preliminary trials to test for evidence that the funnel surface might repel the foraging *Pheidole* ants that were common in the study area. We removed fresh funnel fragments (100–200 mm²) from active nests, placed them with the smoothly brushed inner side up, and baited each center with a small (8–12 mm) paralyzed caterpillar stolen from *Bidentodynerus bicolor* (Saussure) (Vespidae) that were nesting at the same site. Care was taken to handle funnel fragments and caterpillar baits only with forceps. Paired controls were mud fragments of similar size gathered nearby from a naturally cracked dried puddle. Baited fragments and baited controls were arranged in random order along an active ant trail at intervals of about 10 cm and about 2-3 cm to either side of the trail (Figure 5). We exposed the baits simultaneously for 20 minutes and recorded the elapsed time until the caterpillar was discovered and then fully removed from its mud platter.

2.5. Voucher Specimens. Voucher material was compared with authoritatively identified specimens deposited in the CSIRO Insect Collection in Canberra, ACT, and specialists subsequently verified our identifications (see Acknowledgments). Specimens from this study are deposited in the CSIRO Insect Collection; the University of California, Davis (Bohart Museum); the American Museum of Natural History; and the University of Georgia (Fattig Entomology Museum).

FIGURE 5: Experiment to test possible repellency of *A. ephippium* nest funnel to ants (*Pheidole* sp). Funnel pieces were randomly paired with dried mud fragments along either side of a natural foraging trail. Each was baited with paralyzed caterpillars, and their removal by recruited ants was timed. Inset shows ants cooperating to remove a bait.
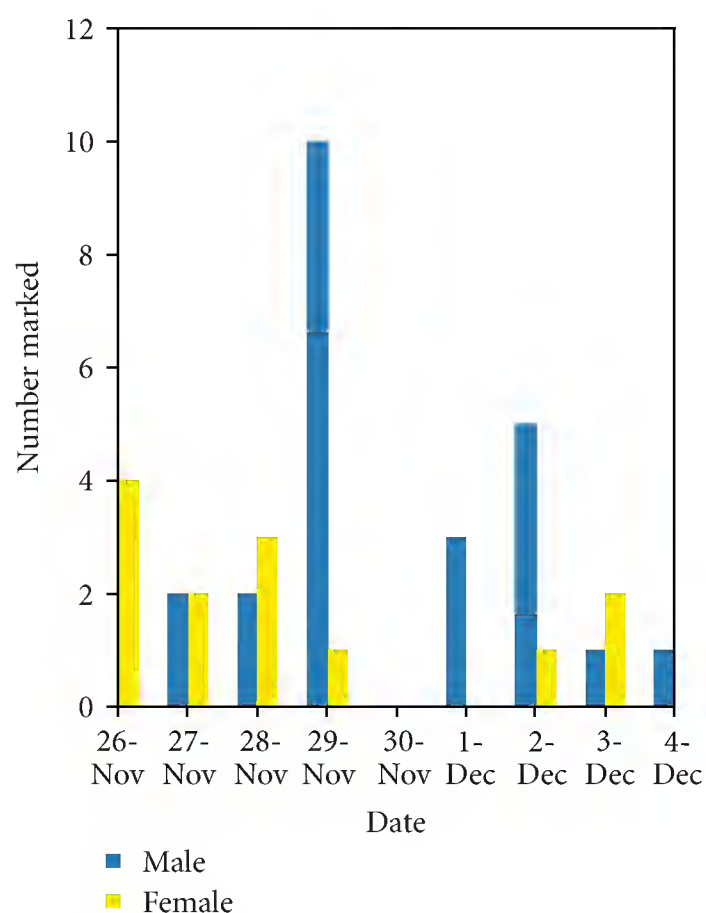

FIGURE 6: Daily number of *A. ephippium* males and females marked at the study site in 2004.

3. Results

3.1. Marked Wasps.

During the 2004 field study, we marked 46 wasps (15 females; 27 males; 4 of undetermined gender) over the course of days (Figure 6). Initial capture was at either our principal study area (18 individuals), the small pool (13 individuals), or nearby buildings monitored periodically (15 individuals). Some of the "cruising" individuals marked, later recaptured, and kept as vouchers subsequently were identified as *A. meadewaldoensis* Perkins, which is morphologically difficult to distinguish from *A. ephippium* (J. M. Carpenter, unpublished), but all nest voucher females were

A. ephippium. Four marked females had active nests under observation, and thus were regularly observed. Another 15 wasps (6 females, 9 males) were seen or recaptured on subsequent days at least once during the study; of these, 6 (2 females, 4 males) were recaptured 2 or more times; one male was recaptured 5 times at 3 different locations.

In 2007, wasp numbers and nesting activity were poor by comparison with 2004. We again captured and marked all wasps seen, but only from the carport area because the pool was now dry. Although we checked other nearby buildings, we did not capture or mark any individuals from those structures. Of the 16 wasps marked at the principal study site, 10 were females and 6 were males. One marked female was regularly seen because she had an active nest. Another 5 wasps (2 females and 3 males) were seen or recaptured on subsequent days—1 male and 1 female reappeared once, 1 female was seen 3 times, and 2 males were seen or recaptured on at least 4 or more days.

3.2. Daily and Seasonal Cycle.

Females were active at nests from just after sunrise until just before sunset. Windy or rainy periods appeared to have little effect on nesting progress. Nights were typically spent away from the nests. A photograph of a single sleeping *Abispa* from Brisbane, QLD, Australia, is posted on the Internet [6], the first such record for this genus; we found no sleeping *Abispa* at our study site.

Depending on nest phase or cell development, females often backed into the funnel and up into the cell to rest motionless with their head facing out, their antennae barely visible. Here they sometimes remained for extended periods (sometimes more than 1 hour). At irregular intervals, they would leave the nest for brief periods, and upon returning usually checked the current cell intensively, often making several inspection circuits around the nest exterior as well.

It appears likely that nesting occurs asynchronously within the *Abispa* population. At least 4 and probably more successive generations occur annually at the study site. We have eyewitness records of adult *A. ephippium* flying around the study site for various dates during every month between September 8 and May 24, which corresponds to all but the very driest "winter" months in northern Australia.

3.3. Nest Location.

Unlike sphecid mason wasps such as *Trypoxylon* or *Pison* that often cluster their nests in suitable places, *Abispa* nests are isolated. In 2004, in an intensive search of an approximately 5-acre area that housed 10 faculty residences with covered outdoor areas that appeared suitable for *Abispa* nests, we located 5 active nests and 7 old nests. In 2007, extensive searching of this same area plus an adjoining area of 15 additional buildings (dormitories, classrooms, and administrative offices) revealed only 2 active nests and 3 old nests. (Some residents reported knocking down nests, however). The only nest found that was not associated with human-built structures was an old nest in a protected tree crevice about 1 m above the ground near the principal study site, but we did not search extensively.

The height above the ground varied. Some nests were within 5 cm of the carport floor; others were among ceiling joists. All nests were attached to 2 adjacent surfaces, such as the junction of an exposed ceiling joist and the ceiling. None was affixed only to a single flat surface. Nests tended to be quite securely attached, tightly conforming to the dimensions of the space. Some occurred in quite unexpected places, for example, in an enclosed space under the seat of a child's plastic motorcycle.

3.4. Nest Construction. A typical *Abispa* nest is built in stages. A cylindrical cell is begun in a relatively dark and hidden corner; the only clue that it is not an inorganic mud blob is the decidedly conspicuous flared funnel that soon extends from its open end (see Figure 1). Average interior dimensions of a cell before funnel construction are 28.7 mm long by 11.8 mm diameter (length range, 23–36 mm; diameter range 9–17 mm; $n = 51$).

Females constructing cells work steadily with little interruption. It took our focal female 4.75 hours and 74 mud-gathering trips to build a single cell to the point of beginning the funnel. Two h and 19 mud trips later, the funnel was constructed. Another 38 minutes were spent at the nest, fine tuning it. The funnel is surprisingly delicate for such a large structure, being less than 1 mm thick at the rim. From an internal diameter of just over an *Abispa* head width (about 9 mm), where it connects to the cell, the bell opening flares to 14–17 mm diameter at its open end; the total length of the funnel is about 20 mm, depending on the point to which one measures.

Inside the empty cell, the female lays an egg. For the next day or 2, she mostly stays within the nest, with her head facing the entrance. Occasionally, she will come out and wander about the nest exterior as though inspecting it. At a time presumed to correspond to egg hatching (in one case, 27.5 hours after apparent oviposition), she leaves and returns with a caterpillar that is placed inside the cell. This may be repeated once or twice. Then she resumes her watchful waiting. Over the next 2–4 days, at decreasing intervals she will bring a few more caterpillars, almost always of the same species and size range. On the final day of provisioning (about 6 days after cell construction), she repeatedly returns with caterpillars, then abruptly stops. This pattern of provisioning is termed truncated progressive provisioning and appears to be characteristic of *Abispa* species [3].

Between provisioning trips, internal nest care is practiced. Periodically and especially toward the end of a cell cycle, *Abispa* undertook episodes of apparent cell cleaning. Repeatedly, she would enter the cell head first, then back part way out through the funnel, sweeping with her foretarsi, and in the process jettisoning bits of debris.

Mud for the final cell closure is obtained by dismantling the funnel, one mouthful at a time. *Abispa* can soften and remove a half-dozen pellets of mud with a single load of water ($x = 6$ pellets, range 5–8); she requires about 30 seconds to remove a pellet and another 30 seconds to reapply it to the cell closure or nest wall. Thus, while dismantling
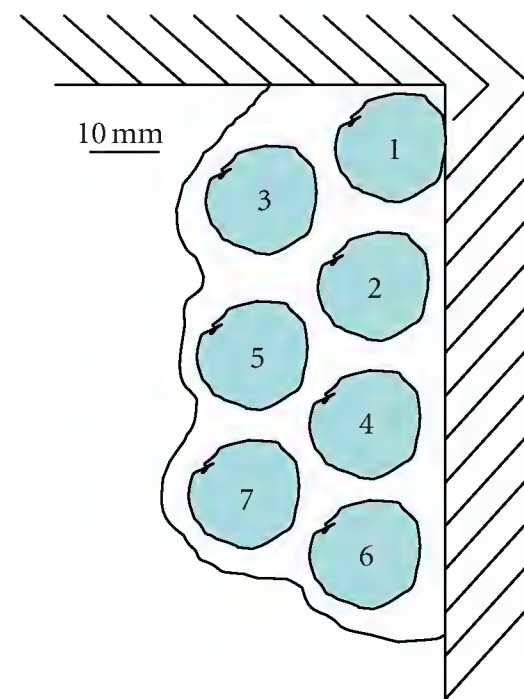

FIGURE 7: Schematic plan of a typical mature *A. ephippium* nest architecture, showing cell arrangement and construction order.

can take almost as long as initial funnel construction, it is time spent almost entirely on the nest rather than away. In timed observations, our focal female in 2004 took 1.5 hours to completely eliminate her funnel, but after the first 45 m the cell was completely closed by funnel mud. She was away from the nest only 19 minutes—4 trips before the cell was closed and other 2 after that for additional water used to obliterate all traces of the funnel. By comparison, collecting the funnel mud involved being away from the nest for 81 m.

At intervals of about a week, construction begins on a new cell as the cycle is repeated again and again. Although some nests are abandoned due to factors such as untimely female death, a complete nest typically has 6–8 cells ($x = 5.6$, SD = 1.9, $n = 15$). New cells are added below, or less typically above, existing cells in a fairly stereotyped order (Figure 7). No nests were more than 2 cells thick, and the greatest number of cells found in any nest was 8. The largest nest exterior dimensions were 80 mm × 54 mm × 38 mm.

When first built, the exterior of a new cell is bumpy, coarse, and quite conspicuous, even after funnel deconstruction. However, at intervals that can span several days, the female alternately inspects the nest exterior and brings numerous mud pellets that she plasters over it, gradually filling in the valleys between cells, thickening the cell wall, and further securing nest edges to the substrate. During these plastering bouts, the female works steadily; after applying each mud pellet, she departs immediately, usually without grooming or further nest inspection. Between bouts, she generally resides within the nest, facing outward; it was during such intervals that we opportunistically conducted nest defense experiments (described hereafter).

Toward the end of the wall-plastering phase, the nest exterior increasingly takes a smooth, shiny appearance as the female works intensively, mouthing a circumscribed area repeatedly. It is generally difficult to detect any liquid being added, but sometimes there is a serpentine wetting that is briefly visible before it dries (Figure 8, arrow). The result is a smooth rounded fortress with walls of variable thickness

FIGURE 8: Female *A. ephippium* applying liquid to nest surface; arrow shows the serpentine drizzle applied most recently by this female.

FIGURE 9: Foretarsus of *A. ephippium* showing ventral tufts of setae on each tarsomere used to polish the interior surface of the entrance funnel.

(range 6–10 mm; $n = 25$ measurements on 8 nests). When nests are broken open, one can usually discern 2 distinct layers; the inner layer represents the initial cell construction, and the outer layer is the added mud plaster (see [3, Figure 3]).

3.5. Mud and Water Foraging. We have used the term "mud foraging" to describe trips that culminated with a female returning with mud in her mandibles. However, like other eumenid mud wasps, *Abispa* does not to collect mud directly but fills her crop with water at one site and makes mud from soil at another location called a quarry [7]. Mud-foraging trips in 2004 averaged 230.4 seconds (range 45–1191, SD = 108.0, $n = 158$). The task of applying the mud load to the nest surface averaged 158.7 seconds (range 21–494, SD = 69.2, $n = 165$).

Once a suitable water source is identified, females appear to return repeatedly to it. One marked female was observed returning repeatedly to drink for 10–30 seconds from the small pool; over a 2-hour period, she made 12 successive trips, returning about every 9 minutes ($x = 540.1$ seconds, range 188–831). On her ninth visit, a male simultaneously arrived and pounced on her immediately; they then flew off *in copulo*. She returned to her drinking spot 707 seconds later.

3.6. Funnel Construction and Maintenance. Funnel construction is a major undertaking. Whereas it took our 2004 focal female 4.75 hours to make a basic cell, it took her another 2 hours (and 19 trips) to construct the basic funnel and an additional 38 minutes to brush it smooth. Forming a large funnel from mud is a significantly more precise task than building a cell; applying each load of mud to build a cell took an average of 131.2 seconds (less than spent on plastering surface mud), but applying mud to the funnel averaged 186.4 seconds ($P = .005$, t test).

During funnel building, a female works steadily, except for the distraction from mating attempts by one or more males that may be present during much of this phase
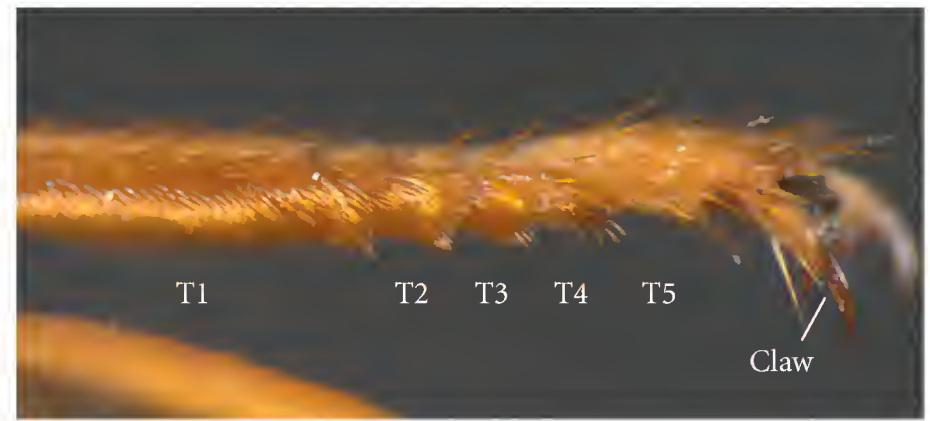
(described hereafter). To make the funnel, a female wasp stands on the nest, gripping it with her mid and hind legs. While her head is inside the incipient funnel bell, her forelegs manipulate the pellet of mud from her jaws. She applies the pellet to the funnel rim, slowly rotating her body in such a way that the rim edge is kept even. At variable intervals she pauses, grips the funnel rim with her middle and hind legs, and synchronously applies her mouthparts extensively to the inner surface of the funnel with a vigorous bouncing movement, a ritual we termed "mouthing."

The smooth, almost polished interior surface of the funnel bell is regularly maintained by brushing with dense tarsal setae unique to *Abispa* females (Figure 9). The brushing ritual encompasses several bouts during which the wasp crawls headfirst into the funnel, then slowly walks backward out to the funnel rim while scraping her foretarsi in unison rapidly and repeatedly over the funnel's inner surface. As she starts each bout in a brushing sequence, she shifts her body slightly around the rim, so that the sequence tracks either clockwise or counterclockwise and sometimes ultimately covers a full 360°.

Complete coverage of the funnel required an average sequence of 9 brushing bouts (range, 7–16, $n = 9$). Funnel maintenance via brushing sequences continues at irregular intervals while oviposition, provisioning, and nest plastering are taking place. There seemed to be no particular external stimulus that initiated brushing bouts, but in the hour following one of our experiments with intruders (described hereafter) brushing frequency noticeably increased. In that instance, the female performed 9 separate extensive brushing sequences in succession, more than what had been seen at any other time. This female also did frequent nest exterior inspections and groomed extensively following the experiments.

To assess *Abispa*'s repair propensities, we purposely removed entrance funnels from 3 active nests. All were at least partially repaired immediately thereafter though the replacement funnels were smaller and less symmetrical than the originals (Figure 10). On another occasion when one side of a nest that originally had been loosely attached to a board was purposely exposed, the resident female switched almost immediately from cell construction and spent most of 2 days plastering mud to cover, smooth, and secure the exposed face (Figure 11). Inspired by this observation, we used an
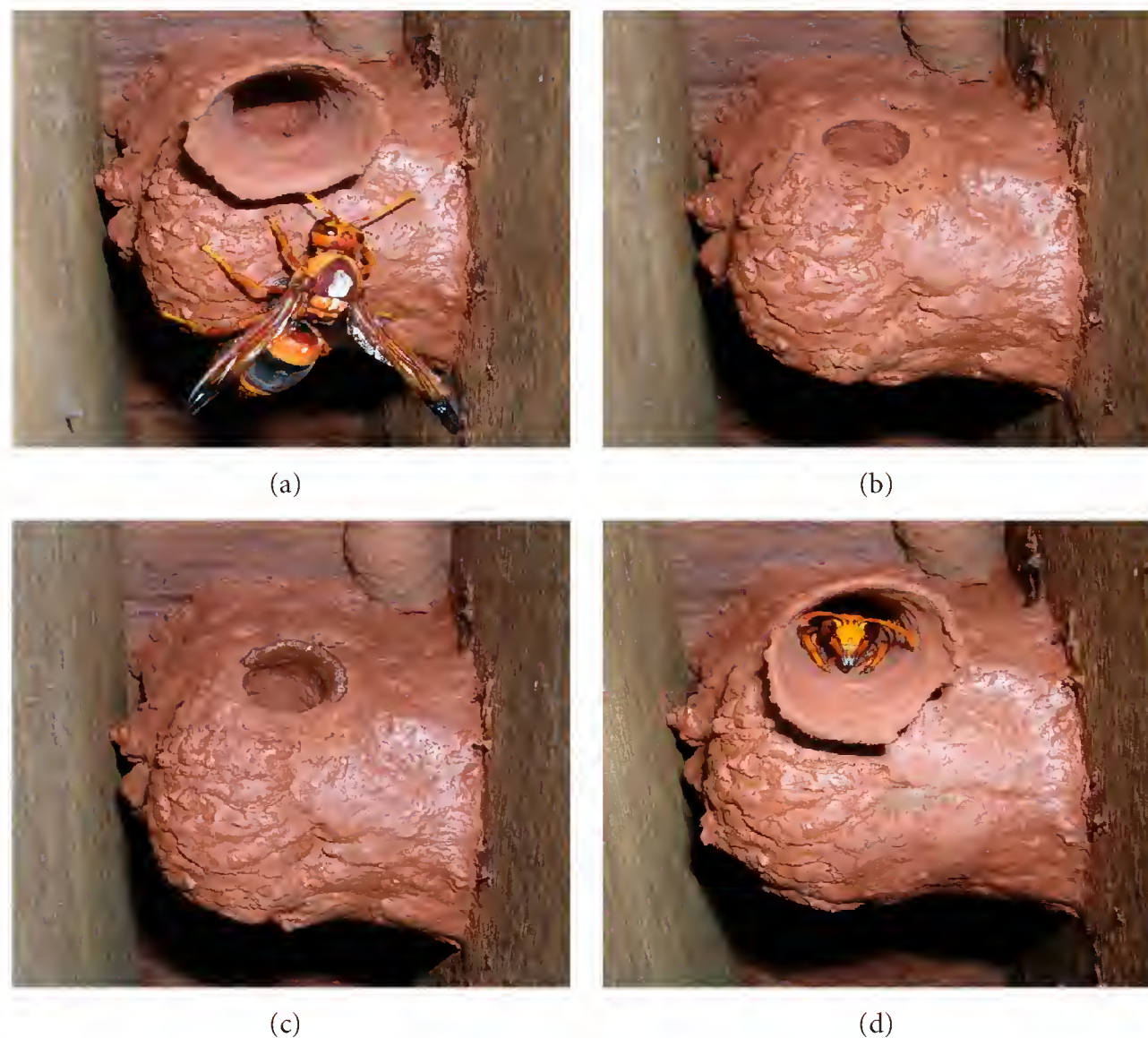
FIGURE 10: Funnel repair by *A. ephippium*: (a) Intact funnel, (b) funnel artificially removed, (c) first mud load applied by female, and (d) completely rebuilt funnel with female in characteristic head-out resting position. Entrance hole diameter 9 mm.
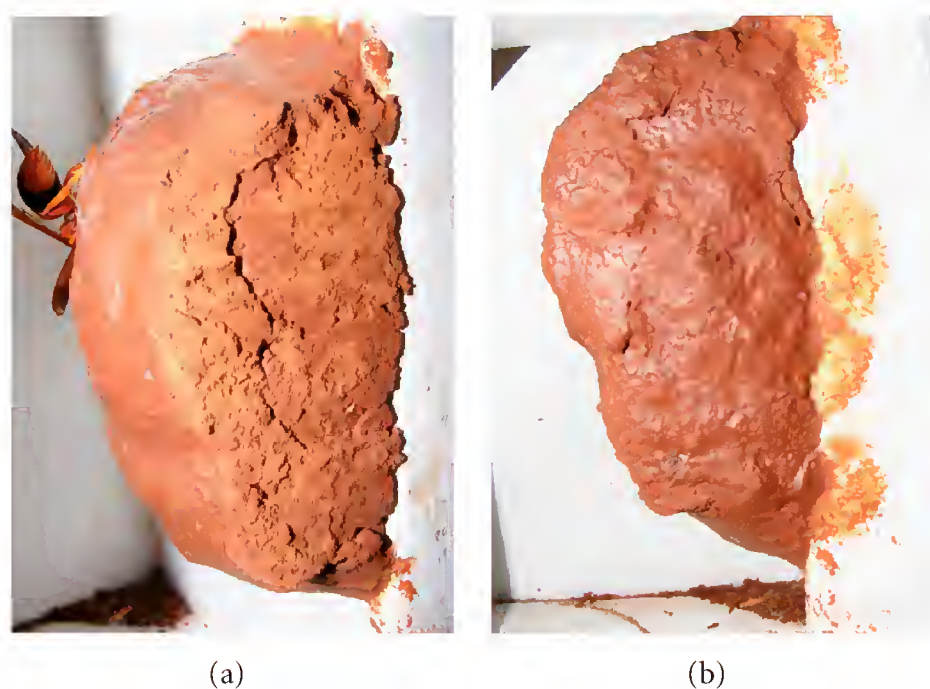

FIGURE 11: Nest before (a) and one day after (b) the front surface of the nest was exposed by removing the board against which it had been built. Note newly added mud over the exposed surface. Nest length 75 mm.

emory board to file a small area on the exterior surface of an active nest to see whether the female would respond to a lesser abrasion; she did not react to the abraded area, but this observation is inconclusive because her exposure to it was relatively brief since for unrelated reasons this turned out to be the last afternoon of her tenure on the nest.

3.7. Prey. Prey foraging involved a considerable investment of time and energy. Thirteen timed trips in which the female returned with a prey caterpillar averaged almost 20 minutes each (x = 1179.7 seconds, range 433–2680, SD = 662.4). Once she arrived back at the nest with prey, the wasp typically spent just under 3 minutes at the nest before leaving again (x = 179 seconds, range 32–466, SD = 141.5, n = 11).

Paralysis of prey is light, and the caterpillars continue to defecate. Prior to pupation, the wasp larva produces a tough parchment-like cell lining that walls off prey fecal pellets and other debris at the distal end of the cell (Figure 12). Two old *Abispa* nests contained cells where the wasp larva had died or failed to develop; 2 prey had pupated in each, and one ultimately eclosed in each, allowing identification of the adults as Pyralidae, probably Phycitinae, and Crambidae, probably Pyraustinae.

Larval prey identifications are difficult because Australian caterpillars are relatively poorly studied. We collected the contents from 2 cells in 2004 and one in 2007 (Figure 13). One cell from a 2004 nest held a large wasp larva and 13 caterpillars of apparently a single species; the other held a large wasp larva and 5 caterpillars that appeared to belong to 2 species. From the 2007 nest cell, we recovered 9 caterpillars of apparently a single species; because this nest was found on the carport floor after falling from the rafters during the night, it originally may have included additional prey.

Food thievery is well known and relatively common among various solitary wasps [8]. We documented prey theft

FIGURE 12: Fragment of opened *A. ephippium* nest showing parchment-like cell lining made by the mature larva. The walled off area shown at the upper end of the cell contained waste materials, cell length 28 mm.

by *Pseudabispa paragioides* (Meade-Waldo) from an active *A. ephippium* nest in 2007. The female came to the nest while the *A. ephippuim* female was away and immediately entered the open cell, exiting quickly a few seconds later carrying a caterpillar.

3.8. Mating. Courtship preliminaries are absent but multiple brief copulations are clearly common ($x = 61$ s, range 42–86, $n = 9$), both at the nest and at water sources. In 2004, males were regularly recorded to visit 3 active nests and several copulations were observed there (Figure 14). The most detailed documentation occurred on the afternoon of May 12, 2004. During the six-hour period in which a female was constructing a new cell and beginning to make a funnel, a single male (green-spot) was continually present and was observed attempting to mount her unsuccessfully at least 7 times. The next morning the funnel was complete by about 0930, but the green-spot male was gone and a new male (2 white spots) was present. The latter was seen to copulate with her successfully 3 times in succession (for 69, 86, and 52 seconds, resp.) between 0940 and 1000, after which he departed. Less than 10 minutes later, the green-spot male arrived, immediately mounted the female, and successfully copulated for 67 seconds. Thus, this female mated at least 4 times with 2 males; probably all were prior to ovipositing in her newly completed cell. Interestingly, 2 days earlier a different marked male had been recorded on this same nest, but no interactions with the female had been observed.

In 2007, the female of our focal nest was observed to copulate 7 times with 3 marked males that visited her nest. One marked male (Figure 14) was observed to mate with her 5 of those times over 2 days as she was completing a new cell and funnel; this same male was seen to approach the nest at least once and usually several times a day over a period of 10 days, including 5 days after the original female had been usurped by *P. paragioides* (described hereafter).

Mating also occurs away from the nest. In 2004, 2 copulations were recorded that were not nest-associated. At 1840 on July 12, 2004, the marked female from our focal nest was netted *in copulo* with an unmarked male as they flew along the rear of the carport. The other occurred the next day at 1545, when a male at the temporary pool mounted a drinking female and the pair flew off *in copulo*.

Nesting females often appeared to simply tolerate mating as a brief interruption of other activities. The one female observed to mate 3 times in succession on her nest with the same marked male and once with a different male simply resumed whatever task she had been doing, seemingly unfazed by the interruption. However, a day earlier this same female repeatedly rebuffed one of the same males' attempts to copulate. Thus females appear to be able to exercise some choice and may not be entirely passive to male mating attempts; receptivity may be modulated by nest stage or ovarian development.

Even though nests are isolated, solitary, and in unpredictable locations, and the nest stage changes cyclically, females at active nests clearly are valuable resources for males. How males locate active nests or whether nest or female odor is involved is unresolved, but males probably learned the location of active nests for they were frequently observed to fly directly to them, approaching and landing with no hesitation. At least some males associate with particular nests for extended periods. In one case, a male was continuously present on a nest throughout much of one day while the female was bringing and applying mud plaster; although the male repeatedly attempted to mount the female, she showed no interest and was not interrupted in applying the mud. This male made at least 6 unsuccessful attempts to couple. Two other marked males remained associated with another nest for extended periods on 2 successive days, again during a period when the female was regularly bringing mud; however, in this case each was seen to successfully mate at least once with the female. If a male was present when a female was "resting" in her funnel (discussed earlier), he often remained for long periods on the exterior of the nest not far from the funnel entrance; however, males were never seen to enter the nest funnel.

On many occasions, individuals flew quickly through the study site without pausing. We counted 149 of these flights during the 10 days of our 2004 study. Most (73.8%) occurred during the morning hours (Figure 15), and the activity peaked in early December. To describe such behavior, a reviewer suggested the term "patrolling," a term generally defined as regular tours of movement to guard or protect a place or maintain order. However, we saw little evidence of such a purpose for these particular flights. Thus, we prefer to provisionally call this behavior "cruising," a recognized slang word for frequenting a public place in search of a sexual partner. Flying speed and the general similarity of the sexes precluded identification of the sex of flying individuals on the wing, but based on captures, most were males. We assumed that they were seeking females, because in a few cases we observed pairs flying *in copulo*. Obviously, resolving the purpose of such flights (and thus the proper terminology to use) will require further study.
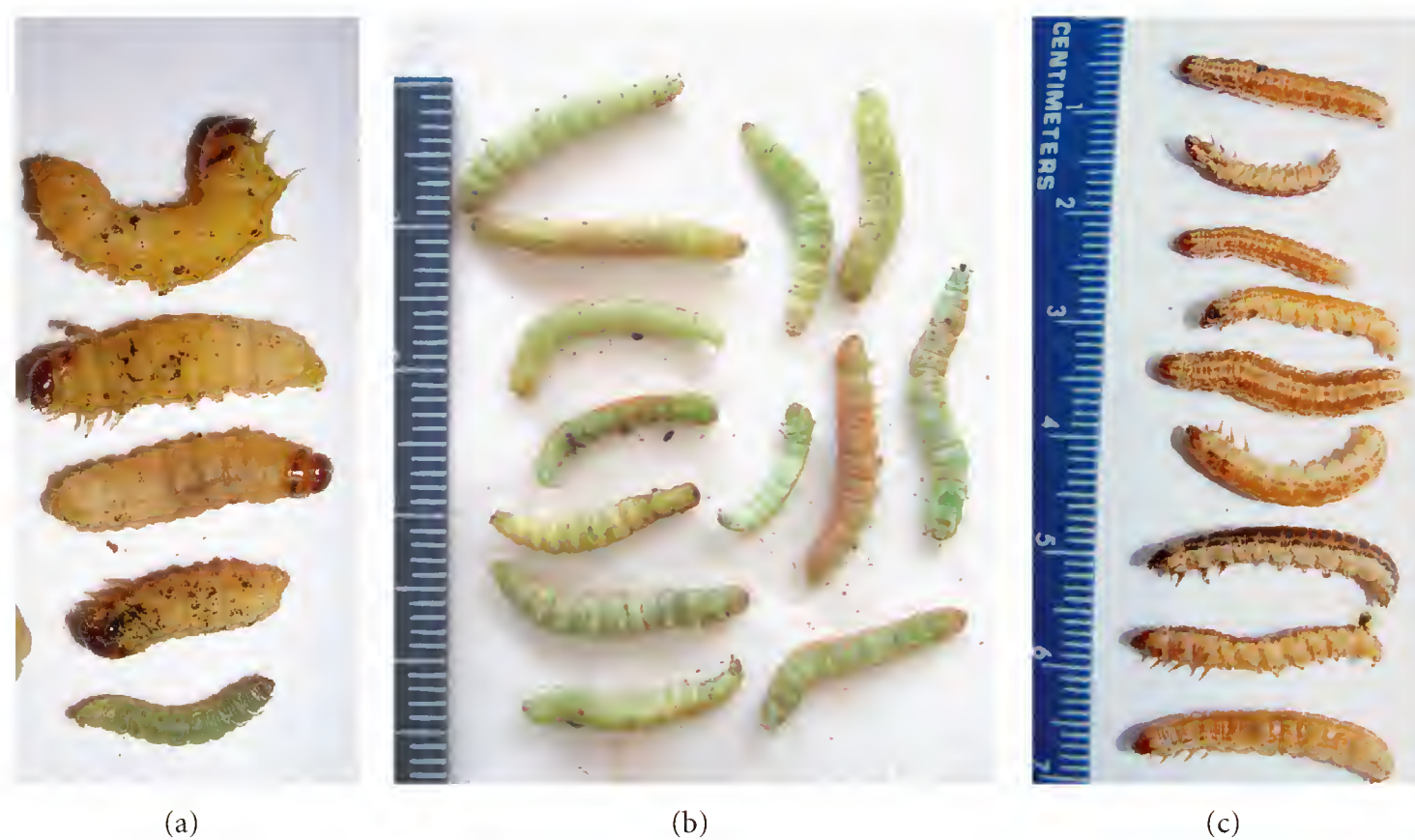
FIGURE 13: Prey from 3 nests of *A. ephippium*. Caterpillars in the left and center frames were removed from nests collected in 2004; those shown in the right frame were from a 2007 nest.
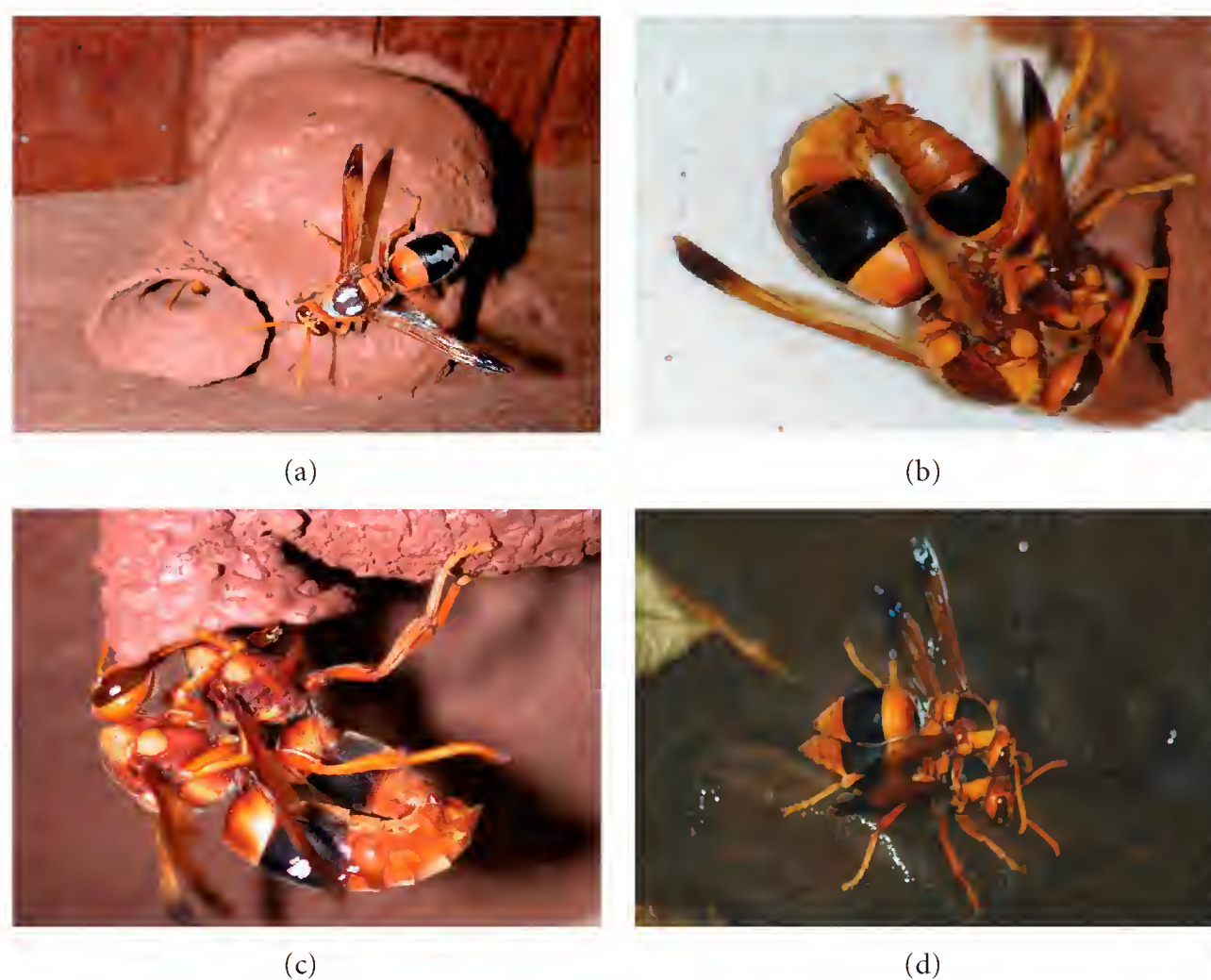
FIGURE 14: Mating in *A. ephippium*. (a) Marked male attending a nest where the female is resting in her nest entrance. (b) Copulation on the nest; note the female's exerted sting. (c) Another pair mating on their nest. (d, courtesy of Bonnie Heim) Male has landed on a female obtaining water at the small pool.

This is not to discount the possibility of territorial behaviors, however. In 2007, the marked male most associated with our focal nest was also repeatedly seen perched at one lower corner of a window air conditioner unit about 15 m from the carport, to which he returned after brief chases of other wasps or periodic flights back and forth along the length of the house. Females visited the AC unit regularly, entering through the grill, presumably to obtain water from the corner of the condensation pan. This male attempted to mate with these females, and was observed to chase and pursue other wasps, probably males. He remained in tenure at the same spot over several successive days. The patrolling behavior occurred intermittently throughout the day, but was more intense in the last hour before sunset; whether

FIGURE 15: Daily number of *A. ephippium* wasps recorded as patrollers or "cruisers" that flew through the study site during the morning hours as compared to afternoons in 2004.
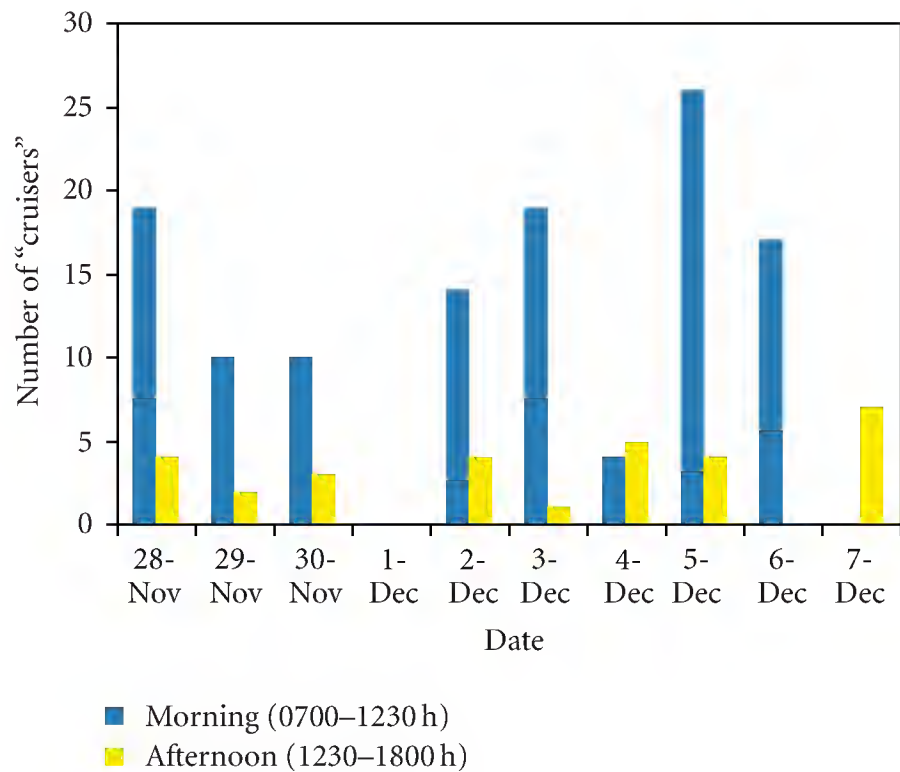
this behavior constitutes true territoriality and therefore an additional *Abispa* male mating strategy also will require additional study.

In 2004, males regularly visited a small temporary pool (see Figure 1) that was frequented by other males of both *A. ephippium* and *A. meadewaldoensis* and by females coming to obtain water. During one 2-hour period, a marked male was recorded to visit the pool 10 separate times. On another occasion we placed 2 freshly killed (by freezing) males in realistic resting positions on decaying mango fruits at the water's edge and watched patrolling males' responses for 3 hours (0900–1200). Although male *Abispa* visited the pool 22 times during this interval, they displayed no obvious interest in the dead males.

3.9. Reuse of Old Nests. Nests often persist for a long time after the original *Abispa* occupants emerge. There is no evidence that *A. ephippium* reuses its old nests or cells of other species' nests. However, cavities left from emerging wasps are highly attractive to various secondary "renters"—bees and wasps that remodel and adapt them for their own use. Six species of secondary nest users were regularly observed at our site: a megachilid bee (*Chalicodoma aethiops* (Smith)), a sphecid wasp (*Pison* sp.), and 4 smaller eumenids (*Paralastor* sp., *Bidentodynerus bicolor,* and *Epiodynerus tamarinus* (Saussure) and *E. nigrocinctus* (Saussure)). Each of these was documented to reuse emerged cells of old *Abispa* nests as well as old *Sceliphron* nests. Biological notes on some of these are to be published elsewhere.

3.10. Development. The 6 mm long egg is suspended from a short (0.5 mm) thread near the distal end of the empty cell (see [3, Figure 4]). Extrapolating from data on emergence times, number of nest cells, and our records for the time to complete a single cell, it appears that (except for the driest

"winter" months of June through August) development from egg to adult requires 4–6 weeks.

Overlapping generations are common in *A. ephippium.* Fairly often, the first progeny complete their development while newer cells of the nest are still under construction. At the 2007 focal nest, at least 2 cells emerged while the nest was still active. In 2004, three nests that were collected while females were still in attendance were found to contain mature pupae or teneral adults. Female eggs are probably laid first; in the 4 cases where we obtained mature offspring, the earliest (oldest) nest cell yielded a female.

3.11. Cleptoparasitism. While the marked resident female at our 2004 focal nest was away from her nest, an unmarked *A. ephippium* female discovered the nest, entered, backed out, reentered, backed out, and then few off. About 2 minutes later she returned and flew directly to the nest, again entering it. The tip of her abdomen was visible over the next several minutes of activity inside the nest. Repeatedly ($n = 6$) she backed partway out, then crawled back in headfirst so that she was entirely inside the cell. Then she backed all the way out, turned around, and backed into the nest so that her head and antennae were all that were visible in the funnel entrance. This newcomer then took up residence, resting partway into the cell with her head facing out inside the funnel, for nearly 4 hours before departing. Curiously, the focal female was away from the nest this entire time—the only time we observed such an extended leave of absence from a nest during active provisioning—and she did not return for the first time until 29 minutes after this unmarked female departed. At first upon her return, her behavior did not appear unusual. However, after briefly resting motionless in the funnel with her head facing out, she moved out onto the nest surface and began 45 minutes of intense activity—making several very thorough inspection circuits, thoroughly brushing the inside surface of the funnel, and occasionally mouthing the funnel's exterior—punctuated with bouts of grooming. Finally, although it was late in the day (1715), she brought in at least 4 caterpillars after this time.

3.12. Nest Usurpation. Several lines of evidence and direct observation, to be published elsewhere, show that female *Pseudabispa paragioides* regularly usurp or supersede *A. ephippium.* These usurpers were common at the study site, especially in 2007 when we captured and marked essentially equal numbers of *A. ephippium* and *P. paragioides* (16 versus 15, resp.).

In 2004, we discovered an active usurped nest with a *P. paragioides* female already present (Figure 16), and recorded 2 *Abispa* nests that contained a cell from which a male *P. paragioides* emerged. There was no way to determine whether the *Abispa* female collected in the 2004 nest that yielded an adult male *P. paragioides* was the original nest-founding female. However, in 2007 we obtained positive proof of the usurpation propensity of *P. paragioides* females and were able to document this usurpation behavior, including vicious and ultimately fatal fighting (Figure 17).
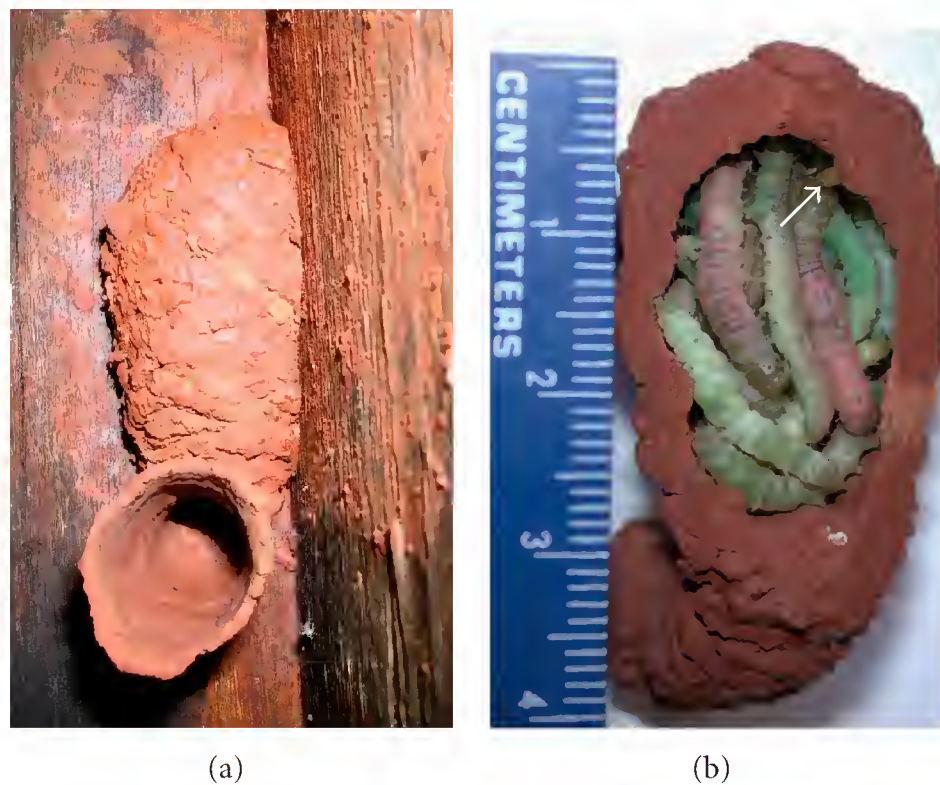
(a)   (b)

FIGURE 16: (a) A single-celled nest of *A. ephippium* that was occupied by a *Pseudabispa paragioides* female when discovered. (b) This same nest was found to contain 20 caterpillars and an egg (arrow), having been mass-provisioned by the *P. paragioides* female.

FIGURE 17: A marked female of *Pseudabispa paragioides* (upper individual) in a battle with a female of *A. ephippium*; shortly after this photograph was taken, the stung *Abispa* female died from the venom of *Pseudabispa* and the latter took over her nest.

3.13. Parasites and Other Nest Enemies. Several relatively ubiquitous parasitoids of other mason wasps such as miltogrammine flies (Sarcophagidae) and *Melittobia* wasps (Eulophidae) commonly gain access to mason wasp nests via the open nest entrance during provisioning. These parasitoids were common at the study site and were reared from nests of other mason wasp species. However, *Abispa* nests at our study site showed a remarkably low level of parasitism. In 2004 none of the 5 active *A. ephippium* nests collected were parasitized, and the only parasitism in 2007 was the chrysidid attack outlined in what follows.

The relatively large chrysidid *Stilbum cyanurum* Forster was common at the study site. In 2007 we observed this chrysidid to land and briefly investigate our focal nest while the female was away. Eight days later at the same nest, the usurper *P. paragioides* (see above) likewise was temporarily

away, having been actively on the nest provisioning a cell 24 minutes earlier. Suddenly a chrysidid appeared and landed on the nest with no hesitation, suggesting previous knowledge of the nest. During subsequent days we recorded what appeared to be the same chrysidid visiting this nest at least once daily, but unfortunately were unable to mark the individual. During one such visit the chrysidid flew back and forth a few cm in front of the nest for nearly 1 minute as though memorizing landmarks, then landed and briefly crawled upon the nest and adjacent substrate for about 12 seconds before flying away. On another occasion, the chrysidid approached as the resident *P. paragioides* was actively mouthing an area on the lower part of her nest; it did not attempt to land but flew off after only 10 seconds with no evident notice from the resident female.

At the time of its attack, *S. cyanurum* first walked about briefly on the lower (older) nest surface, but quickly focused on a small area and began to chew into the mud at a location that upon nest dissection proved to be cell 1 with a late stage *Abispa* pupa (Figure 18, left). After just over 1 minute of chewing, the parasite reversed position and thrust her ovipositor into the newly made hole (Figure 18, right). Oviposition was completed within 15 seconds, after which she again reversed position and began to refill the hole with mud chips. The entire process lasted about 9 minutes. When the *Pseudabispa* female arrived 10 seconds later, she did not initially detect the chrysidid, which had retreated to the bottom of the nest, but within 30 seconds she began to patrol the nest surface and upon encountering the chrysidid, immediately chased it away. As soon as she departed on another provisioning trip, however, the chrysidid returned and spent another 9 minutes filling the rest of its oviposition hole before finally departing. Later nest dissection revealed that in addition to the parasitism of cell 1 that we observed, a second cell immediately above it had also been parasitized and contained a similar-sized chrysidid larva feeding on the host prepupa.

Lizards and spiders are likely to be common enemies of mud wasps; dead wasp mummies were regularly seen in old spider webs at the study site. Large huntsman spiders (*Holconia* sp.) also frequented the rafters and individuals were seen near nests, but no predation by or upon these arachnids was observed. An Asian house gecko (*Hemidactylus frenatus* Schlegel) rested adjacent to one active *Abispa* nest for 3 consecutive days. It seemed not to notice the comings and goings of the relatively large wasp; likewise, the resident wasp paid no obvious attention to it.

3.14. Challenge Presentations. Do *Abispa* actively defend their nests, and if so, how? Because parasitic bombyliid flies were commonly seen at our study site and are known to parasitize many solitary wasps and bees [7], we glued freshly killed bombyliid flies (*Thraxan* sp.) to flexible palm frond fiber straws (see Figure 3, b) and presented one to a female resting inside her nest funnel, head facing out, at the midpoint of a cell construction cycle. She quickly lunged out, decapitated the fly in an instant, then retreated into the nest. A second fly presented minutes later also elicited a similarly

FIGURE 18: *Stilbum cyanurum* attacking a nest of *A. ephippium*. The parasite chews a hole through the nest wall (a), inserts her telescoping abdomen into the hole to oviposit (b), and refills the hole with mud. Inset shows the chrysidid larva consuming an *Abispa ephippium* pupa found in the cell upon later dissection.

rapid response; she grappled with this fly and removed its right wing. After the second encounter, the female briefly flew off the nest but returned to take up her resting position in the cell entrance after 143 seconds. Over the next hour she performed 9 separate bouts of funnel brushing. Then we exposed her to 3 more similarly presented flies sequentially over about 10 minutes. Each fly was viciously attacked, bitten and partially dismembered, after which the female retreated to rest in her nest entrance, head facing outward, never once flying from the nest.

Three days later, we repeated these experiments at 2 other nests and again at the focal nest when the female was constructing a new cell. At the first of these nests, the female was resting inside. When challenged by a more or less stationary fly as before, she responded immediately by lunging repeatedly with opened mandibles, flying off briefly, and then returning to inspect the nest exterior. We challenged her a second time, wiggling the straw such that the fly appeared to hover immediately in front of her. At this, she responded with a brief wing buzz but no lunge or bite. As we continued the challenge, she did too, buzzing briefly 4 to 5 times in rapid succession. After 52 seconds with no physical contact, we removed the fly. After 12 minutes, we wiggled the fly at her once again. She immediately lunged, then wing-buzzed 3 times, twice, then 3 more times. Five more times we presented a jiggling fly, each time eliciting whirring wing buzzes until, after 57 seconds, she flew.

At the second nest, the female was away but a male visitor was resting on the exterior. When challenged with the wiggling fly on a straw, the male immediately backed away to hide behind the funnel, then came to the front side of the nest and took flight. There was no evidence of any nest defense, and the male was gone for 25 minutes before returning.

The final set of presentations with a fly on a straw was performed back at the original focal nest, where the female

was now in the early stages of constructing another cell. Her response was swift and "lethal" to 2 bombyliids offered in succession, and included biting off parts of their wings.

How would *Abispa* behave if a potential intruder were already present? Two days later, while the female was away we glued another freshly killed *Thraxan* sp. fly directly onto a nest below the funnel (see Figure 4). Returning with prey, the female apparently saw it, but with full jaws she crawled over the fly 3 times on her way into the funnel to place her prey in the cell. She then departed and returned with nothing after 8 minutes. This time upon coming to the fly she bit at it repeatedly; however, she was unsuccessful in dislodging it. After walking around the nest exterior as though inspecting for other intruders, brushing the funnel interior, and grooming a bit, she backed into the funnel. Over the next 65 minutes, the female left the funnel and approached the fly 7 more times, each time lunging repeatedly (8–19X, $n = 3$) without dislodging it, then groomed, brushed, and inspected in no particular sequence, and returned back into the funnel for increasingly long periods between bouts. An hour after these interactions, we removed the fly from the nest; it was "shopworn" but still intact.

We duplicated this procedure the same day at another nest with essentially similar results. In this case, the female was resting deep within the nest when we glued the fly on, and she immediately noticed it as she came out. She repeatedly alternated inspection circuits, short flights off the nest, circling the parasite, and lunging at it. She did not enter her funnel again until 12 minutes after initial contact, but then backed in, came out and lunged, and then backed in again to block the entrance. At this point, we removed the fly.

Given the violence of these attacks against dummy flies approaching or on the nest, it is probably unsurprising to note that later nest dissections showed no evidence of successful parasitism by bombyliid flies, despite their abundance at the study site and the fact that they were regularly observed flying along the rafters. These parasitic flies oviposit near solitary wasp nests, not directly within them. The *Abispa* female's frequent cell cleaning episodes probably prevented this parasite's larvae from successfully invading the cell.

Responses to freshly killed cuckoo wasps presented to wasps at the same 2 nests and in the same fashion as bombyliids also elicited strong responses (see Figure 3, (a)). For example, the first and second times that she was confronted by a cuckoo wasp inserted part way into the funnel, the female lunged at the intruder several times with such force that it could be felt through the straw. She bit at the intruder vigorously at each lunge, but it remained undamaged. Following a third confrontation, *Abispa* retreated deeply within her funnel, her head blocking the cell entrance; despite being actively nudged several times with the tethered parasite, she did not budge.

What factors release such aggressive behavior? We glued a small freshly killed *Ropalidia* worker to a straw and similarly presented it to our focal female just as she was finishing plastering some mud to the nest exterior. In 5 trials, each
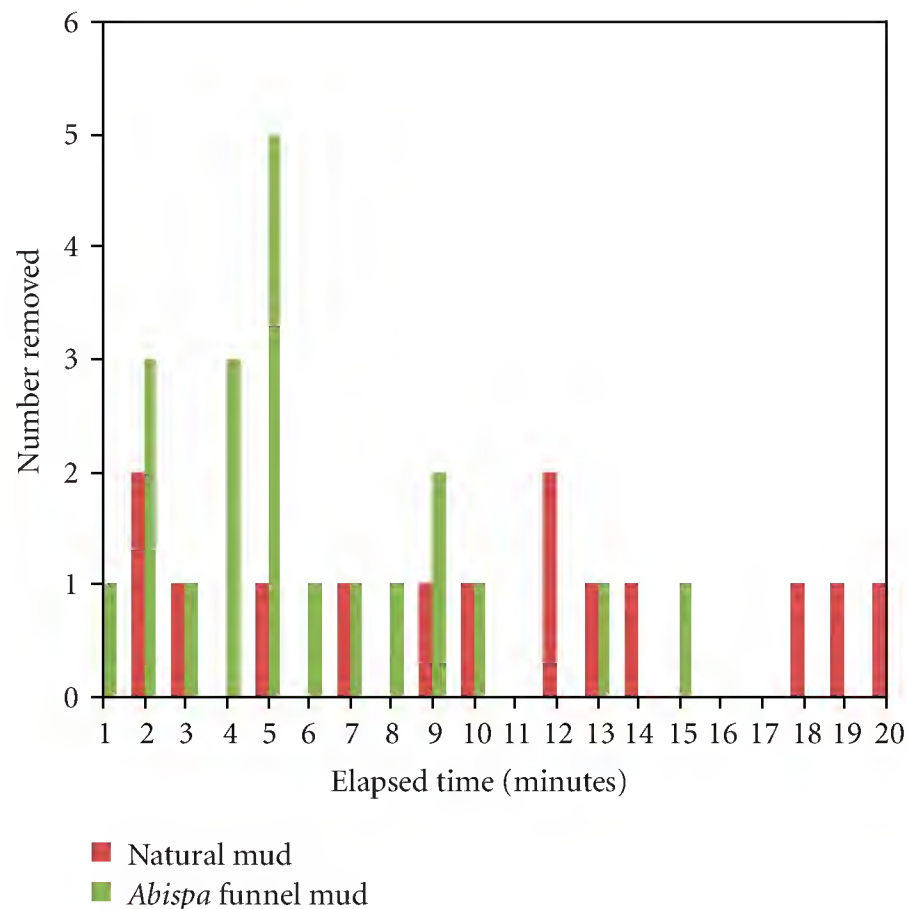
FIGURE 19: Number of caterpillar baits removed from pieces of *A. ephippium* funnel mud and control mud pieces over a 20-minute interval.

lasting approximately 1 minute, she repeatedly lunged at the intruder but did not make actual physical contact. Then we presented a freshly killed *Sceliphron formosum* and (as a rough indication of visual rather than chemical cues) a "wasp" model of the same size constructed of 3 small colored beads threaded on a paperclip, each presented 4 times in succession for 1 minute, with about 2 minutes between trials. The female's responses did not differ discernibly between the two actual wasp bodies and the bead-wasp; however, during the fourth trial with the bead model, she only lunged once. As with our other experimental manipulations, these were admittedly crude trials, done *ad hoc* under field conditions, but they seem to suggest that for *Abispa* visual cues such as a potential intruder's size and apparent motion are more important than odor cues in threat recognition.

3.15. Does the Nest Funnel Deter Ants? Although others [9] have postulated that the funnel's purpose is to protect nest contents against ants, paralyzed caterpillar baits were actually removed faster from the funnel pieces (x = 325.4 seconds, SD = 217.6) than from the field mud pieces (x = 455.5 seconds, SD = 404.3) and overall slightly more were removed from funnel pieces (21 versus 19), suggesting that wasp-made mud might even be attractive to foraging ants but certainly contained no repellents. However, as graphed data clearly illustrate (Figure 19), these differences were not statistically significant (P = .2225, t-test). Whether the frequently-brushed interior of the funnel acts as a physical "ant slide" would be an interesting topic for further study.

4. Discussion

Most aspects of *Abispa ephippium* nest structure and basic biology agree with those reported elsewhere for *A. ephippium* [4] and *A. australiana* [3]. Thick-walled nests are highly dispersed and lifetime female fecundity appears to be low (<10) with a correspondingly low immature mortality rate. Nest construction is slow, requiring approximately a week for each cell; coupled with the observed cell cleaning episodes and responses to potential parasites, this provides evidence for extended parental care.

Based on mark/recapture data, the overall sex ratio in the study population appeared to be male-biased. However, this may be an artifact, in that females tend to be nest-oriented and we located relatively few active nests whereas male mating strategies (described hereafter) predispose them to be more frequently encountered. Also, we discovered after the study concluded that some patrolling males were *A. meadewaldoensis*.

An *Abispa* nest with a stock of paralyzed prey is a valuable resource for a potential usurper, predator, or parasite. For a usurper, these equate to time and energy savings and reduced vulnerability to dangers associated with foraging. Like other solitary wasps, *A. ephippium* females are vulnerable to usurpation and parasitism because by necessity they must spend considerable periods of time away, leaving their nest undefended.

We documented that *P. paragioides* females not only usurp nests but also steal prey from partially provisioned cells of unsuspecting *A. ephippium* females while the latter are away from their nests. It is clear that *Pseudabispa* females regularly use existing *Abispa* nests and may obligatively do so. No unequivocal *Pseudabispa* nests were ever found despite extensive searching in 2 seasons.

It is generally believed [7] that cleptoparasitism has not evolved among solitary vespids as it has among sphecid wasps and bees, yet we observed behavior suggesting the possibility that an *Abispa* intruder entered a nest, destroyed the owner's egg or newly hatched larva, then laid her own egg. This may be the first report of this behavior among the solitary Vespidae. Unfortunately, our evidence is circumstantial since we did not remove this nest until another week had passed.

Parasitism incidence was extremely low compared to levels experienced by many other solitary nesting wasps [10]. The only confirmed parasitism was the 2 cells in the focal 2007 nest that contained larvae of the cuckoo wasp *Stilbum cyanurum*. *Stilbum* species are reported to attack nearly every species of mud-nesting wasp in the Old World [11]. To date this is the only parasite recorded for *A. ephippium*. However, the bombyliid fly, *Thraxan* sp., used for challenge, was common at our study site, and although we did not confirm it to be parasitic on *A. ephippium,* it has been reared from nests of a related species, *A. splendida* [12].

The extraordinary thick wall that characterizes *Abispa* nests is a formidable barrier representing a considerable investment of parental effort. Its importance as a parasite deterrent was first suggested upon finding a very low incidence of parasitized cells in nests of a related species, *A. australiana*. Of 21 cells in 7 nests in that study [3], only 2

were parasitized, also by a chrysidid. In the present study parasitism occurred after the demise of the nesting female during nest takeover by *Pseudabispa*.

The extended parental care—including inspection, patrolling, active nest defense, and cell cleaning—practiced by female *A. ephippium* during the progressive provisioning of each successive cell appears to have restricted the nest's vulnerability to parasitism to the point in the nesting cycle where parental activity is waning or absent. Experimental results with other solitary nest-building wasps [13] also indicate that an extended period of parental attendance at the nest renders offspring less vulnerable to parasite attacks.

Our challenge presentations are the first indication of parental nest defense in *Abispa*. Females' vigorously aggressive attacks upon freshly killed cuckoo wasps, *Sceliphron*, *Ropalidia*, and even inanimate bead models demonstrated the active role females play in protecting their nests.

Because of the ubiquity and well-known polyphagy of *Melittobia* (Hymenoptera: Eulophidae) [14], we expected to find these tiny wasps parasitizing *Abispa*. However, although we confirmed the presence of *M. australica* Girault at our study site as a frequent parasite of other wasp and bee species and we confirmed that prepupae of *A. ephippium* were suitable and acceptable hosts (unpublished observations), *M. australica* was never found to have successfully parasitized this *Abispa* species. Likewise, unidentified satellite flies (Diptera: Sarcophagidae) were commonly seen at the study site. We never saw them pursue *A. ephippium*, and only once did we observe them following a *P. paragioides* female that usurped a cell in an *Abispa* nest; we later recovered 2 small maggots from among the prey in that cell. We suggest that the repeated cell inspections and cell cleaning practiced by *A. ephippium* females, in concert with truncated progressive provisioning, serve to minimize parasitism by these two common enemies of other solitary mud dauber wasps.

Construction of solitary isolated nests rather than clustered nests in groups is a third factor that probably contributes to the minimal parasitism experienced by *A. ephippium*. Evidence from other solitary wasps [7, 10] indicates that nest clumping may increase parasitism rates as well as prey theft by conspecifics.

Male mating behavior in *A. ephippium* has been studied previously for a population in New South Wales [5]. In the present study, multiple recaptures reconfirmed that *Abispa* males are highly mobile, appearing at different sites over several days. In the New South Wales study, male *A. ephippium* patrolled pools along an intermittently flowing stream and mated with females that arrived to obtain water. Our *A. ephippium* males also did not defend their water source, even though our pool was much smaller than the pools in the former study.

Based on recaptures and observations of marked individuals, it was clear that *A. ephippium* males practice more than one tactic in their efforts to locate receptive females. First, they regularly cruised through the study site where females were actively nesting. Second, they periodically visited and patrolled the small nearby pool where many females came to obtain water. Third, certain males were observed to visit and associate with particular active nests on a regular basis (see

Figure 14). Finally, possible defense of a point water resource (air conditioning unit) was also observed. It appeared that all of these tactics were conditional strategies practiced by individual males at different times, depending on local conditions. All strategies also appeared to be successful.

Copulations were most often seen at focal nests where we spent the bulk of our observation time, and copulation also was recorded at the small pool and a coupled pair was seen in flight at the study site. Mating on nests is recorded for both *A. ephippium* [4] and *A. splendida* [5]. With multiple copulations observed between marked individuals, our study has also confirmed the multiple mating previously described for *A. ephippium* [5]. Copulation duration ($x = 61$ seconds) and the absence of courtship preliminaries were also comparable to previous reports for *A. ephippium* [5].

Because hunting trips entail females being absent from the nest, often for extended periods, male presence on nests could potentially deter parasites such as cuckoo wasps that appear to "trap-line" nests, visiting them at regular intervals to assess their potential for parasitism. In a brief preliminary trial we presented a nest-associated male with a dead cuckoo wasp glued on a straw. His response was initially to back away to the far side of the nest and then to fly, suggesting that males play little role in nest defense. In other contexts, nest-associated males may provide some deterrent against nest enemies, as we once observed a nest-associated male to lunge at a potential *P. paragioides* nest usurper, causing her to fly off.

Chimney turrets and entrance funnels are constructed by many other solitary nesting vespids, but few are as spectacular as the flared funnel entrance to *Abispa* nests. This downward projecting bell is so conspicuous as to be like a beacon advertising the doorway to a potential bonanza for predators, parasites, and competitors/usurpers able to cue into it. Various hypotheses have been advanced regarding the possible functions of turrets and entrance funnels, but none have been experimentally tested [7]. Our preliminary experiments that confronted females with freshly killed bombyliid and chrysidid parasites showed that the funnel gives *Abispa* females the advantage of blocking the entrance while simultaneously mounting a quick, strong attack upon invaders in a confined arena. While we never observed a nest enemy attempting to walk upon the slippery funnel surface, chrysidids have been observed losing their purchase and sliding down the funnels of a soil-nesting mason wasp, *Paralaster* sp. [15].

We showed that *A. ephippium* females immediately repair and restore damaged funnels (see Figure 10), indicating that the entrance funnel likely plays some important role. Funnel brushing frequency also dramatically increased following our intruder experiments, an observation that strongly hints at an intruder deterrent role for the funnel. Whether the well-developed foretarsal brushes (see Figure 9) apply some substance to the inner surface or simply act to polish it and maintain its smoothness needs further investigation. The discovery that the antennae of certain *Philanthus* wasps (Crabronidae) secrete symbiotic bacteria that inhibit fungus growth [16] suggests another potential area of investigation that might be fruitful.

Although a funnel would provide increased surface area for dispensing chemicals, our experiments with a common local ant species failed to support a chemical deterrency hypothesis. However, as Cowan [7] notes, "the full impact of ants on solitary wasps is probably underappreciated; unlike many other enemies, they may be in a nest for only a short time, and they leave no evidence (such as cocoons) of their visit." Furthermore, experiments with other nest parasites and predators are needed before we can rule out the existence of possible allelochemical roles in other contexts.

If the funnel were serving primarily to broadcast some deterrent chemical, one might expect it to be left in place at the end of nest construction, even though *Abispa* plugs the entrance hole at its base. However, *Abispa* removes the funnel completely at each cell's completion and builds it anew for each subsequent cell. This observation suggests that whatever the funnel's purpose, it must serve primarily during the interval when the egg has been laid and the cell is being provisioned.

Funnel deconstruction does provide a readily available supply of mud that can be used to close a completed cell rapidly at a very vulnerable stage of nest construction. Collecting this mud at an earlier point in the cell construction cycle entails much lower risk of parasitism, usurpation, and prey theft. Reusing funnel mud for cell closure requires only a few brief water-gathering trips, minimizing and greatly lowering the time (by 77% in our study) that the nest must be left unguarded when the cell is fully provisioned with a developing larva.

Speculating further, we note that humans commonly use funnel-shaped guards above or below bird feeders to thwart squirrels. Lizards have many behavioral and structural features similar to squirrels, so perhaps the funnel functions as a deterrent for lizards or other larger carnivores by making it difficult to insert tongues far enough into the cell to extract prey or larvae. Lizards occurred near nests at our study site, and such predators might be even more common at more natural *Abispa* nesting sites such as within rock crevices.

Acknowledgments

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Research Article

Prevalence of Stylopization of *Sphex ichneumoneus* (L.) (Hymenoptera: Sphecidae) by *Paraxenos westwoodi* (Templeton) (Strepsiptera: Xenidae)

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On a seed production farm in southcentral Montana, USA, we found the strepsipteran *Paraxenos westwoodi* (Templeton) parasitizing adult *Sphex ichneumoneus* (L.), which were collected while they were foraging for nectar. Over a two-year period, 25% of males and 7% of female wasps were stylopized, as evidenced by the presence of puparia and empty puparial cases of male and female *P. westwoodi* exserted dorsally between abdominal segments. Our estimate is based on a sample size larger than those usually reported for strepsipterans attacking solitary aculeate wasps. We review the literature on strepsipteran prevalence in solitary aculeate wasps and provide an updated list of solitary wasps known to act of strepsipteran hosts in North America.

1. Introduction

In North America, Strepsiptera of the families Stylopidae and Xenidae parasitize adult aculeate bees and wasps (Hymenoptera: Apoidea) [1–4]. Species of five genera of Stylopidae (*Crawfordia*, *Eurostylops*, *Halictoxenos*, *Hylecthrus*, *Stylops*) attack solitary bees of the families Andrenidae, Colletidae, and Halictidae. Species of two genera of Xenidae attack Vespidae, with *Polistes* (Polistinae) hosting *Xenos* and eumenine wasps of at least five genera *Pseudoxenos*. Finally, species of the xenid genus *Paraxenos* parasitize solitary wasps of the family Sphecidae. Strepsiptera that attack Hymenoptera have long been known to cause profound sublethal effects, including effective castration, but death of the adult host bee or wasp is delayed until late in the life cycle of the strepsipteran [4–7].

Salt [5, 6] described external morphological changes associated with stylopization in females of three species of *Ammophila* (Sphecidae). The most common modifications included altered patterns of pubescence on the head and thorax and reduced length of spines on the legs, with the result that those female characteristics had become more male-like. Recently, Tatsuta and Makino [8] found that

overwintering queens of *Vespa analis* (F.) parasitized by *Xenos moutoni* were unable to reproduce the following season. Kudô et al. [9] reported that *Xenos myrapetrus* (Trois) caused infected workers to be smaller than uninfected workers of *Polybia paulista*.

Hymenopteran host records for strepsiterans are often based on a few individual infected wasps found in samples collected for other purposes. As a result, little information exists on the prevalence of parasitism within host populations of solitary wasps. Here, we report on rates of parasitism by *Paraxenos westwoodi* (Templeton) in *Sphex ichneumoneus* in a sample of 182 wasps collected at one site over a two-year period in southcentral Montana, USA.

2. Materials and Methods

On nine days from 3 July to 11 August 2006 and three days from 17 July to 7 August 2007, we collected 182 *S. ichneumoneus* adults at the USDA-NRCS Bridger Plant Materials Center (BPMC), 4 km southeast of Bridger, Carbon Co, Montana. The vast majority of the wasps were collected either individually or within general sweep samples on flowers of slender white prairie clover (*Dalea candida* Michx.

TABLE 1: Prevalence of *Pseudoxenos westwoodi* parasitism of *S. ichneumoneus* at the Bridger Plant Materials Center, Bridger, MT.

	Sex of host	2006	2007	Both years
Number of wasps examined	both sexes	55	127	182
Stylopized	male	4	6	10
	female	0	10	10
Not stylopized	male	11	19	30
	female	40	92	132
Prevalence of stylopization (%)	male	26.7	24.0	25.0
	female	0	9.8	7.0
	both sexes	7.3	12.6	11.0

FIGURE 1: (a) Posterior dorsum of female *Sphex ichneumoneus* showing two *Paraxenos* exserted at posterior border of gastral tergum IV. On the left is a female puparium, and on the right is the remains of a male puparium following the adult's emergence. (b) Male puparium prior to emergence of adult on another host individual.

ex Willd.), which is grown in monoculture at BPMC for seed production. A few wasps were also taken in pan trap samples within stands of *D. candida*; several others were collected in nets on snowberry (*Symphoricarpos* sp.) or sowthistle (*Sonchus* sp.). All specimens were returned live under ice to Montana State University on the day of capture, and then either frozen or placed in vials for rearing of the strepsipterans in vials at room temperature. All wasps were examined under a stereomicroscope for the external presence of strepsipterans exserted between gastral segments; because we did not dissect hosts to check for the internal presence of triungulin larvae, it is possible that we have underestimated prevalence of stylopization. Voucher specimens of the host wasp and the strepsipterans have been placed in the Montana Entomology Collection, Montana State University, Bozeman.

3. Results and Discussion

Based on comparison to the species description, the strepsiterans parasitizing *Sphex ichneumoneus* were identified as *Paraxenos westwoodi* (Templeton); *Pseudoxenos smithii* (Heyden), previously recorded as a parasite of this wasp [2], is a synonym of *P. westwoodi*. Overall, 11% of *S. ichneumoneus* adults examined had evidence of stylopization (Table 1). On 16 wasps, one or more *P. westwoodi* were present as puparia or adults exserted from the host's gaster. On eight wasps, we found evidence that one ($N = 7$) or two males had exserted and then emerged from the host (Figure 1). Among the 20 parasitized wasps, seven males and seven females harbored one puparium, whereas three males and three females carried two puparia each. One of the 26 puparia or adult xenids protruded from the membrane following gastral segment III, 15 were positioned after segment IV, and 10 followed segment V. Twelve were located just left of the center of the longitudinal axis of the body, 13 were just to the right of center, and one was far to the right of center, though still between two tergal sclerites. On two wasps, triungulins (instar I larvae) were in the process of emerging from female puparia when they were preserved.
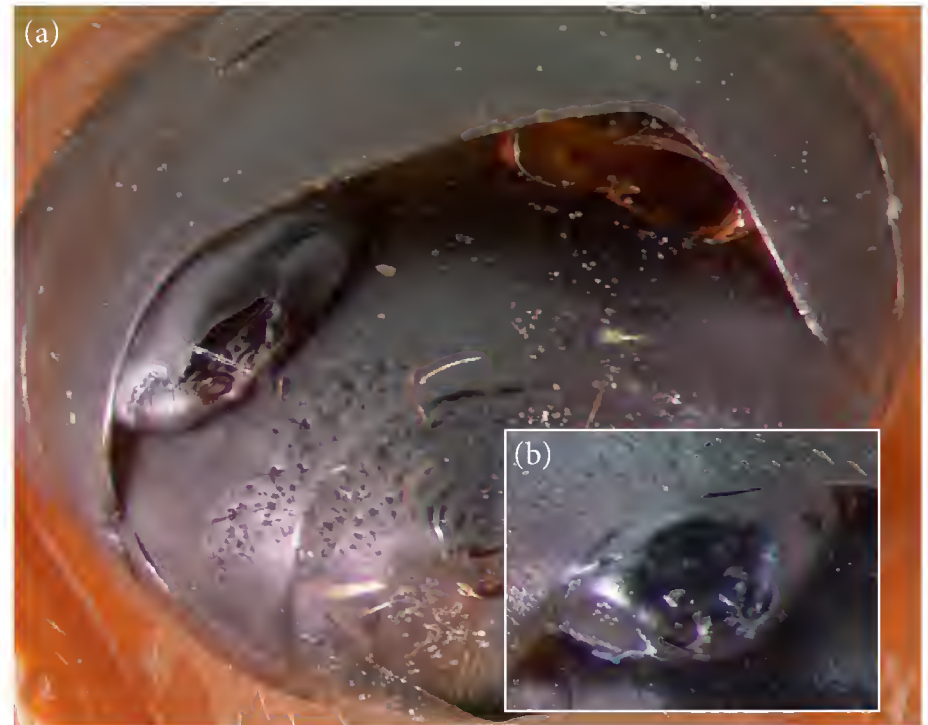
The sex ratio of the adult *P. westwoodi* (15 males : 11 females) did not differ significantly from 1 : 1 (chi-square goodness-of-fit test, $\chi^2 = 0.34$, $P = .56$, d.f. = 1). However, the prevalence of parasitism among all *S. ichneumoneus* collected (independent of the number of parasites per wasp) was 3.5 times higher in host males than females (chi-square contingency table, $\chi^2 = 10.29$, $P = .0013$, d.f. = 1). Although this could be due to a real sex bias in rates of parasitism, our data cannot be used falsify the alternative hypothesis that stylopized females were less likely than males to be collected foraging on flowers, where almost all of our samples were obtained. Salt [6] indicates that stylopized hymenopteran females have a lower propensity to forage. If this is the case for *S. ichneumoneus*, our sample may not only misrepresent sex bias in primary attack rates, but it may underestimate the overall rate of parasitism. On the other hand, if stylopized wasps are easier to collect than unparasitized individuals, if they are weaker fliers for example, then we may be overestimating the incidence of parasitism. Another potential source of bias could have occurred if parasitized *S. ichneumoneus* had longer life spans than unparasitized individuals, as can be the case for other species of insects [4].

Prevalence differed between years for females ($\chi^2 = 4.22$, $P = .04$, d.f. = 1), but not for males ($\chi^2 = 0.04$, $P = .85$, d.f. = 1). However, the overall prevalence did not change significantly from 2006 to 2007 ($\chi^2 = 1.11$, $P = .29$, d.f. = 1).

The range of previously reported rates of parasitism of solitary aculeate wasps by strepsipterans encompasses the overall value of 11% that we observed. Clausen [10] cites Piel [11] recording a 25% stylopization rate in *Isodontia nigella* (F. Smith) (= *Sphex nigellus* F. Smith); Kifune and Yamane [12] and Kifune [13] report that *I. nigella* is stylopized by *Paraxenos esakii* (Hirashima and Kifune). Evans and

TABLE 2: North American species of Sphecidae known to be parasitized by Strepsiptera (based on records from Krombein et al. [2] and Kathirithamby and Taylor [3].

Strepsipteran species	Wasp host species
Paraxenos lugubris (Pierce)	*Ammophila aberti* Haldemann, *Ammophila breviceps* Smith, *Ammophila evansi* Menke, *Ammophila nasalis* Provancher, *Ammophila pictipennis* Walsh, *Ammophila pruinosa* Cresson, *Ammophila urnaria* Dahlbom, *Eremnophila aureonotata* (Cameron)
Paraxenos auripedis (Pierce)	*Isodontia auripes* (Fernald)
Paraxenos luctuosae (Pierce)	*Podalonia argentifrons* (Smith), *Podalonia luctuosa* (Smith), *Podalonia violaceipennis* (Smith)
Paraxenos duryi (Pierce)	*Prionyx atratus* Lepeletier
Paraxenos westwoodi (Heyden)	*Sphex flavovestitus* Smith, *Sphex habenus* Say, *Sphex ichneumoneus* L., *Sphex pensylvanicus* L.

Matthews [14] reported rates of stylopization of 8% in 250 *Bembix variabilis* Smith and 12% in 320 *Bembix littoralis* Turner in Australia. Krombein and Van der Vecht [15] found that 3 of 10 *Bembix orientalis* Handlirsch in Sri Lanka were stylopized by *Paraxenos krombeinii* Kifune and Hirashima.

Rates of strepsiteran parasitism have also been reported for several solitary Vespidae (Eumeninae). In Japan, Itino [16] documented an 8% stylopization rate in *Anterhynchium flavomarginatum* Smith; earlier, Iwata [17] had reported *Pseudoxenos iwati* Esaki as a parasite of this wasp. *Pseudoxenos hookeri* (Pierce) was present in 33% of nests and on 10% of individuals of *Euodynerus foraminatus apopkensis* (Robertson) in the United States [18]. In Australia, 13% of 54 male and 25% of 24 female *Paragia decipiens* Shuckard (Vespidae: Masarinae) were stylopized, an overall prevalence of 17% [18]. It seems likely that even lower rates of strepsipteran parasitism than reported above are more typical of infected populations, but that they often remain unquantified or unreported. Therefore, we encourage all authors that report records of stylopization to include information on the number of hosts examined, the method by which they were collected, and the time period over which hosts were obtained.

In Table 2, we combine the information from previous sources [2, 3] to provide a list of sphecid host records for Xenidae in North America; the table includes updated taxonomic information on both hosts and parasites. In North America, *Paraxenos* species apparently specialize *P. lugubris* on *Ammophila* and the related genus *Eremnophila*, *P. luctuosae* on *Podalonia*, and *P. westwoodi* on *Sphex*. Outside of North America, species of *Paraxenos* attack sphecids, including *Sceliphron* [19], and crabronids (e.g., *Bembix*, *Tachytes*) [12, 14, 15, 20].

Despite the fact that the behavior and ecology of solitary wasps and their natural enemies are well studied in North America (for reviews see [21, 22]), we actually know very little about the effects of Strepsiptera on individual host fitness or the population dynamics of host species in this region. Reports, such as that given here, at least provide some evidence that, in certain years and locations, *Paraxenos* can parasitize a significant proportion of a host population. However, long-term studies that include life-table analyses [23] of the host populations would be required to more fully explore the effect of *P. westwoodi* on *S. ichneumoneus*.

Acknowledgments

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Review Article

Evolutionary Aspects of Acaricide-Resistance Development in Spider Mites

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Although the development of acaricide resistance in spider mites is a long-standing issue in agricultural fields, recent problems with acaricide resistance may be characterized by the development of complex- and/or multiresistance to acaricides in distinct classes. Such complexity of resistance is not likely to be a single mechanism. Pesticide resistance involves the microevolution of arthropod pests, and population genetics underlies the evolution. In this review, we address the genetic mechanisms of acaricide resistance evolution. We discuss genetic diversity and linkage of resistance genes, relationships between mite habitat and dispersal, and the effect of dispersal on population genetic structure and the dynamics of resistance genes. Finally, we attempt to present a comprehensive view of acaricide resistance evolution and suggest risks under globalization as well as possible approaches to managing acaricide resistance evolution or emergence.

1. Introduction

Safe and sufficient agricultural production is one of the most important global issues of the 21st century. Along the demands of food safety and minimizing environmental impacts, the idea of pest management is rapidly moving from chemical control toward sustainable technologies involving the exploitation of ecological functions. Nevertheless, insect pests and diseases cause huge yield losses in crops production, and pesticides have played an important role in mitigating yield loss of agricultural produce. Considering that the occurrence and geographical distribution of pests may change with global warming and economic globalization, pesticides will continue to be important in future agricultural production.

However, repeated pesticide use results in microevolution due to the selection pressure ultimately leading to resistance development. Marked development of acaricide resistance has been found in spider mites (Acari: Tetranychidae), which have short generation and high propagation potential. Since the 1990s, many populations of the two-spotted spider mite, *Tetranychus urticae* Koch, and the European

red mite, *Panonychus ulmi* (Koch), have developed global resistance against many new acaricides. Among the top 20 resistant agricultural and medical arthropod pests, ranked by number of unique compounds in Arthropod Pesticide Resistance Database (http://www.pesticideresistance.org/), the most resistance has developed in *T. urticae*, whereas *P. ulmi* is the ninth most resistant [1].

Recent acaricide resistance problems are characterized by the development of complex- and/or multiresistance to acaricides in distinct classes. Pleiotropic effects may explain multiresistance in that a single key factor, for example, *trans*-acting factors, confers resistance to multiple insecticides involving distinct classes via a simultaneous increase in the action of several metabolic mechanisms involved in resistance [2]. Similarly, acaricide selection frequently confers cross resistance to acaricides in the distinct classes on spider mites, for example, *T. urticae* [3, 4] and *P. ulmi* [5]. However, the mechanisms of such resistance are largely unknown.

The complexity of acaricide resistance found today may not be explained by a single mechanism. *Tetranychus urticae* is a ubiquitous species that vigorously attacks an enormous number of plant species. Cross-resistance and the inheritance

of resistance are not necessarily common to localities in this mite. These findings suggest that each selection process possibly leads to a different type of resistance mechanism, depending on the location and past selection history, and may yield different cross-resistance patterns.

Pesticide-resistance development is an example of microevolution in arthropod pests. Population genetics, which is affected by many factors including biological and ecological traits of arthropod pests, properties of pesticides, and pesticide application patterns [6–8], underlies the evolution. In this review, we address genetic mechanisms during the process of acaricide resistance evolution and discuss features of resistance genes and their genetic linkage, relationships between habitat and mite dispersal, and the effect of dispersal on population genetic structure and the dynamics of resistance genes to gain insight into spider mite resistance management in the global physical distribution environment. Finally, we comprehensively evaluate the evolution of acaricide resistance and suggest the global risks and management prospects.

2. Aspects and Features of Acaricide Resistance

Today's acaricide resistances assume a new complicated aspect rather than the past; that is, genetic mode of resistance is regionally different. Etoxazole resistance is inherited maternally in Korean *T. urticae* populations [9], implying that it is caused by a mutation in cytoplasmic mitochondrial DNA. In contrast, in Japanese populations, the etoxazole resistance locus is located on a nuclear chromosome [10]. Hexythiazox resistance is under monogenic control in Australian populations [11], whereas more than one locus is involved in Japanese populations [10]. Similarly, chlorfenapyr resistance in Belgian populations is under polygenic control [12, 13], but Japanese populations might have a resistant allele at only one of the possible chlorfenapyr resistance loci (monogenic) [14]. Moreover, while clofentezine resistance confers a high level of cross-resistance to hexythiazox [15] but not to etoxazole in Australia, hexythiazox resistance probably confers etoxazole resistance in Japanese local populations [10, 16]. Cross-resistance has not been reported between etoxazole and hexythiazox [17, 18].

Monogenic resistance is favored under field selection regimes [19–21], and acaricide resistance is typically monogenic [11, 22–24], although some instances of polygenic resistance have been reported [25–27]. However, each polygenic allele for chlorphenapyr and hexythiazox resistance may be capable of conferring substantial resistance [10, 12, 13]. Previous standard polygenic inheritance descriptions likely cannot adequately predict the evolutionary process of this type of inheritance in which several or a few resistance genes have additive and major effects on the resistant phenotype. In this regard, the LC_{50} for hexythiazox is notably higher for polygenic resistance in Japan (>10,000 mg L^{-1}) [10] than for monogenic resistance in Australia (48 mg L^{-1}) [11], whereas similar LC_{50} values for chlorfenapyr were reported in a Japanese resistant population (2,130 mg L^{-1})
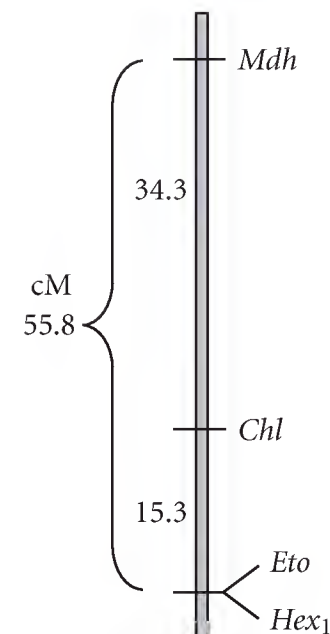

FIGURE 1: Gene map of a linkage group of loci for malate dehydrogenase (*Mdh*), resistance to chlorfenapyr (*Chl*), etoxazole (*Eto*), and hexythiazox (*Hex$_1$*) in *T. urticae*.

[14] and a Belgian resistant population (2,939 mg L^{-1}) [12]. Moreover, the potential for each resistance gene is individually associated with other distinctive resistance genes.

3. Genetic Linkage among Acaricide Resistance Genes

Most spider mite species have arrhenotokous parthenogenesis; males develop from unfertilized eggs (haploid), and females develop from fertilized eggs (diploid) [28]. This type of parthenogenesis may favor the fixation of fewer but more advantageous alleles [29, 30] but see [31, 32] such as acaricide resistance alleles under acaricide application. Fast fixation of advantageous alleles in male haploids may result in rapid fixation of linked alleles [33], as in the hitchhiking effect [34, 35].

To date, little is known about the genetic linkages among acaricide resistance genes. Uesugi et al. [14] revealed that *T. urticae* etoxazole and chlorfenapyr resistance genes are genetically linked to each other with a recombination rate of 14.8% (15.3 cM) using the three-point cross with malate-dehydrogenase allozymes [36] and located at 55.8 and 34.3 cM from loci accompanying etoxazole and chlorfenapyr resistance, respectively (Figure 1). They predicted the probability of hitchhiking effects between two closely linked acaricide resistance genes under acaricide selection, calling this phenomenon "apparent cross-resistance." Although hexythiazox resistance is under monogenic control in Australian *T. urticae* populations, Asahara et al. [10] demonstrated that more than one locus is involved in hexythiazox resistance in a Japanese *T. urticae* population. Moreover, they found that one of the loci associated with hexythiazox resistance was tightly or completely linked to the etoxazole resistance locus (Figure 1). This suggests that the development of hexythiazox resistance possibly conferred etoxazole resistance in a Japanese local population of *T. urticae* that had never been exposed to etoxazole application [16].

Construction of a genetic linkage map is helpful to determine the effects of genetic relationships among loci associated with distinct acaricides, including the hitchhiking effects during the development of acaricide resistance, as Yan et al. [37] analyzed in yellow fever mosquitoes, *Aedes aegypti* (L.) (Diptera: Culicidae). Genetic markers, especially microsatellites, should be useful tools for genetic mapping of resistance genes. A substantial number of microsatellites have been cloned and analyzed in spider mites, including the citrus red mite *Panonychus citri* (McGregor) [38], *T. urticae* [39–41], and the Kanzawa spider mite *Tetranychus kanzawai* Kishida [42]. Microsatellites have also been applied to kin species such as *Tetranychus turkestani* (Ugarov and Nikolskii) [43]. We conduct a linkage analysis of these microsatellites and have revealed two (12 loci) and three (14 loci) linkage groups in *T. urticae* and *T. kanzawai*, respectively (Uesugi et al. unpublished data, Matsuo et al. unpublished data). A linkage map with higher resolution should be complete in the near future.

4. Breeding Patch and Genetic Differentiation

Incidental factors such as gene flow (migration rate) and founder effects affect the dynamics of the local frequency of resistance genes in a metapopulation. Follett and Roderick [44] demonstrated that several resistance foci formed in a metapopulation through model analyses in which migration followed a two-dimensional stepping-stone model under the assumption that individuals move only to neighboring subpopulations in a latticed distribution and a percentage of the populations go extinct at each generation. Spider mites are tiny, wingless arthropods. They exhibit two major dispersal behaviors, crawling and aerial dispersal [45–47]. Crawling is assumed to be used over short distances, such as between leaves within a plant, whereas aerial dispersal may be used for longer distance migration.

We addressed spider mite movement and breeding patches on a host plant because there is some evidence of sibmating in *Tetranychus* spider mites: a fertilized female deposits many eggs on the same leaf and very little migration occurs during development under uncrowded conditions [48–52]. Hinomoto and Takafuji [53, 54] studied the genetic structure of a *T. urticae* population on strawberries based on phosphoglucoisomerase allozyme frequency [36]. Interestingly, they found the highest level of genetic differentiation, as measured by the fixation index (F_{ST}) [55], at the smallest habitat (leaflet) level. F_{ST} decreased with increasing the habitat size, that is, leaflet > leaf > plant [53, 54]. In contrast, the value of the inbreeding coefficient (F_{IS}) [55] was the highest at the largest habitat level (subdivided section in the strawberry beds) and the lowest at the smallest habitat level (leaflets) [53]. In this regard, the Wahlund effect probably became larger in larger habitat sizes pooled for F-statistics analyses. Therefore, such genetic structure implies that although *T. urticae* females and males randomly mate on breeding patches (leaflets), gene flow among the patches is limited. The tendency to form breeding patches probably increases when population densities are low [50, 52] and such genetic structure may often cause genetic differentiation

among breeding patches through random genetic drift and/or founder effects.

Adult *P. citri* females are rather mobile; their microhabitat is greenish twigs with foliage in citrus orchards and the females deposit their eggs on plural leaves [56]. Osakabe and Komazaki [57] focused on the genetic structure in a limited habitat area and performed several laboratory experiments with small citrus seedlings using esterase locus (α-*Est1*) alleles [58]. They introduced females from two *P. citri* strains, in which the α-*Est1* locus was fixed to distinct alleles on the small citrus seedlings, and analyzed phenotype frequencies. As a result, although the inbreeding coefficients within a seedling decreased with population increase, the inbreeding coefficients remained substantially higher than zero even after the mite population had expanded enough to overlap the introduced colonies. *Panonychus citri* may couple randomly by nature, at least within the smallest breeding patch of a single leaf [57]. Despite their expected mobility [56], adult *P. citri* females oviposited on very few leaves, at least when the population density was low, and most individuals stayed on the leaves where they had been oviposited during the juvenile developmental stages in the experiments by Osakabe and Komazaki [57].

Adult males usually guard quiescent deutonymphal females and copulate with newly emerged females immediately after their last molts [59]. Once the first copulation for a virgin female has been completed, subsequent mating with other males is ineffective [60, 61]. Many offspring mature on the same leaf, at least during low population density. These spider mite habits might facilitate founder effect in each breeding patch and influence preservation (or fixation) of rare genes, such as acaricide resistance genes.

Under a condition of no acaicide selection, Uesugi et al. [62] demonstrated that genetic drift also works on the dynamics of an acaricide resistance allele; they found a remarkable decrease in mortality following an etoxazole application in two of 32 experimental subpopulations isolated for 13 generations, whereas the resistance did not develop in another 64 subpopulations that experienced limited gene flow.

5. Dispersal Depending on Habitat Structures and Environments

Dispersal behavior, colonization, and the resulting population structure of spider mites should strongly affect the distribution and spread of acaricide resistance genes [63]. Because of vigorous reproduction, spider mites cause leaf and plant deterioration, resulting in both food shortage and desiccation [64, 65]. Such unfavorable conditions motivate dispersal behaviors.

Dispersal, which is an important event for settlement of new habitats [63, 66–68], may be affected by the surroundings of microhabitats that may vary depending on the agricultural system, for example, between woody and herbaceous plants and between greenhouse and field situations. This should result in genetic structure diversity of each local population and a consequent geographic distribution of alleles. Goka and Takafuji [69] found that

Japanese *T. urticae* populations on fruit trees and roses could be subdivided into three geographical groups based on allozyme loci allele frequencies, whereas no particular geographical patterns were detectable in populations that inhabited annually herbaceous crops.

5.1. Dispersal and Genetic Differentiation in Greenhouses. Most local populations on herbaceous crops occur only temporarily. Thus a population increase may begin by invasion of a small number of mites, facilitating founder effects and genetic differentiation between local populations even if their host plants are adjacently located [69, 70]. Particularly under greenhouse conditions, population growth on herbaceous crops usually starts with a small number of immigrants. They may often experience an intensive bottleneck due to acaricide application in a closed place. This is also the case for greenhouse-grown perennial plants such as roses, although spider mites may maintain their populations on host plants throughout the year. Spider mites are unlikely to use aerial dispersal by means of wind-borne in a greenhouse due to the calm conditions. Windborne dispersal requires adequate wind velocity of 1.5 m/s or more [71–73]. Instead, dispersal by crawling or passive transportation by human movements through agricultural practices is expected in greenhouses [40].

Under such restricted dispersal conditions, genetic differentiation is likely to occur among greenhouse colonies, especially at low population densities [50, 52]. Hinomoto and Takafuji [53] found significant genetic differentiation in a *T. urticae* population on greenhouse strawberries (F_{ST} between sites = 0.16, F_{ST} between plants = 0.35). Tsagkarakou et al. [70] investigated population structures in greenhouses in France, where roses, carnations, egg plants, and tomatoes were cultivated. They also found significant differentiation within a greenhouse (F_{ST} between sites = 0.01–0.33). In contrast, Navajas et al. [40] obtained low estimates of F_{ST} (0.008-0.09). Nevertheless, the latter findings may not contradict the former examples. Navajas et al. [40] sampled mites from areas incontrovertibly wider than the breeding colony ranges; for example, 15 eggplants were sampled from each site (25 m long transects), and two females per plant were used for the analysis. Such sampling strategies might obscure the effects of genetic differentiation among breeding patches on the population genetic structure data. However, their data demonstrated a general trend toward significant heterozygote deficiency [40], suggesting a Wahlund effect due to substantial isolation of the mite population into smaller breeding patches.

Uesugi et al. [74] analyzed the fine-scaled genetic structure of *T. urticae* exposed to more than one acaricide heavily applied to rose trees in a greenhouse. They set 18 consecutive quadrats (length: 1.2 m) in two beds (lengths: 22 and 27 m) in a greenhouse and analyzed the genetic structure using microsatellites. As a result, the level of genetic differentiation (θ) [75] among quadrats was higher in the bed with lower mite density (0.221–0.34) than in that with higher mite density (0.134–0.14) [74]. An analysis of molecular variance (AMOVA) revealed a temporal change in allelic frequencies in the bed with lower mite density ($P <$

.001), but not in that with higher mite density [74]. These differences likely reflect the larger genetic drift at lower mite density; small population size could cause strong genetic drift, thereby decreasing genetic variation, which is indexed by allelic richness and heterozygosity, and increasing genetic differentiation among quadrat populations [76, 77]. Uesugi et al. [74] found that isolation-by-distance [78], calculated by comparing the $F_{ST}/(1-F_{ST})$ values with the spatial distance of each quadrat population [79], was significant in the bed with higher mite density (Mantel test, $P < .05$) but not in that with lower mite density. A significant positive autocorrelation was detected by spatial autocorrelation analysis [80] only within a short range (2.4–3.6 m) of the bed with higher mite density, suggesting that migration was limited to a short range, whereas larger-scale dispersal, such as aerial dispersal, did not appear to contribute to the genetic structure [74].

Spider mites within a breeding patch are likely to mate randomly rather than assortatively, and an increase in population density effectively reduces F_{ST} values among patches [54, 57]. Nevertheless, fine-scale genetic structure is preserved under stable environments such as a perennial host plant in a greenhouse [74]. Yano [81] demonstrated collective dispersal and resulting aggregation at a new feeding site in *T. urticae*; crawling dispersers tended to follow the trails of former dispersers, which may be associated with the threads spun by spider mites. Therefore, the tendency for high F_{IS} values in greenhouse spider mites may be attributed to the traits of spider mites forming breeding patches on small habitats, such as an individual leaf, and the limitation of immigration among patches. Consequently, gene flow between breeding patches is delayed even if mites are located on the same plant. Instead, founder effects and genetic drift promote genetic differentiation between breeding patches via fixation to specific alleles in each breeding patch.

5.2. Aerial Dispersal Mode and Gene Flow in Orchards and Citrus Groves. Spider mites aerially disperse by means of wind-borne or ballooning. Young gravid adult females are the dominant dispersers in both aerial dispersal means [82, 83]. However, species that exploit the wind-borne and ballooning probably achieve different arrival distances. In a practical investigation, dispersal of only 10–20 m occurred from blackberry into corn [66, 71]. Model analyses have suggested 5.5–12 m and 16–48 m dispersal from corn and apple, respectively [84]. In peanut field experiments by Boykin and Campbell [71], regardless of release height for aerial dispersal, most *T. urticae* caught by sticky traps were only 60 cm above the ground (the lowest traps) and catch numbers decreased as the traps were set higher (the highest traps were set 2.1 m above the ground). Therefore, as a general rule for the passive aerial ground dispersal, the number of arrivals per unit area probably declines steeply with increasing distance from the point of departure [85]. In contrast, in the field experiments by Fleschner et al. [82], ballooning *O. punicae* were captured at the top 60 cm of a 6 m (5 × 5 cm) pole in an avocado grove, suggesting that they drifted upward after takeoff and that mites dispersing by ballooning migrate longer distances than do windborne mites. In fact, *P. citri* has been observed aerially migrating

between adjacent citrus groves [82] and from distant citrus groves to Japanese pear orchards [86]. Almost imperceptible air currents are sufficient for mites to balloon [82].

Uesugi et al. [87] investigated the structures of *T. urticae* populations in two apple orchards using five microsatellite loci. In the first orchard, apple trees were planted in lines at intervals of 5–8 m, and their branches did not contact neighboring trees. In the second orchard, apple trees were planted at intervals of 2 and 7.5 m within and between lines, respectively. To exclude effects on gene flow from direct crawling dispersal between apple trees, Uesugi et al. sampled mites from apple trees along a diagonal transect in the second orchard. Ultimately, mites were collected from apple trees separated by distances of 10–24 m within about 100 m of the lines in these orchards. As a result, heterozygote deficiency (f) [75] and θ among trees were positive in these orchards ($f = 0.328$ and $\theta = 0.028$ in the first orchard, and $f = 0.078$ and $\theta = 0.012$ in the second orchard). This suggests that there was a certain level of limitation to mite migration, and complete genetic homogenization was prevented within a scale of the studied sites. Nevertheless, analyses of genetic similarity and spatial location using dendrograms, isolation-by-distance, and multiallelic correlograms revealed no significant genetic differentiation among trees in these orchards. This may be explained that the level of gene flow is relatively high over long distances of <100 m, resulting in no genetic structure among mite populations in distant trees. Similarly, Grafton-Cardwell et al. [63] found that the frequencies of *T. urticae* and *Tetranychus pacificus* McGregor resistant to cyhexatin and dicofol were equivalent in on neighboring almonds and cotton, although cyhexatin had been applied only on almonds while dicofol had been applied only on cotton, indicating the occurrence of substantial gene flow between mite populations on these plants by aerial dispersal. Notwithstanding, significant isolation-by-distance was found in an analysis of wider geographic scales (populations were 10 m to more than 100 km apart) using allozymes in Greece [88]. Those suggest limitations for this mite to regionally disperse using the wind.

Genetic differentiation among geographically distant populations was also found in *P. citri*. Osakabe et al. [89] analyzed *P. citri* genetic structure hierarchically from the level between patches within a tree to between regions (Honshu and Kyushu islands of Japan), using the coefficient of gene differentiation (G_{ST}) [90, 91]. They found that G_{ST} tended to be smaller with decreasing area and distance between populations [89]. This geographic trend was consistent with the results of a protein analysis by Osakabe and Sakagami [92]. However, the diversity of *P. citri* populations within a local population and/or a microhabitat was different from that of *T. urticae*, which maintained frequent gene flow in a local population [87, 93]. The divergence within a grove and/or a tree clearly accounted for the total diversity of *P. citri* populations; G_{ST} values between trees within a grove and between patches within a tree were larger than those between groves. These results suggest that *P. citri* frequently migrates between citrus groves within a locality but not across wider areas. Moreover, higher diversity among trees (or patches) within groves than between groves implies

that this mite migrates mainly by aerial drift (ballooning) between groves [82], whereas crawling dispersal is limited. Thus interbreeding between patches is infrequent and solely dependent on interpatch distance [57].

Consequently, the two modes of aerial dispersal in spider mites, wind-borne and ballooning, likely result in widely different population structures. Dispersion patterns of species exploiting the wind-borne might be explained using an aerodynamic model, such as the seed flux model [94, 95], as described by Jung and Croft [84]. These patterns may represent a practical decline in the number of arrivals per unit area with increasing distance from departure point [85]. The significance of microhabitat for *P. citri* genetic population structures was also demonstrated in marine organisms with reduced dispersal, such as polychaetes, which disperse only during the planktonic larval stage [96].

6. Effects of Dispersal and Habitat Stability on Acaricide Resistance Development

Patch size and persistence of host plants may largely influence population biology as well as gene flow between patches (colonies) and/or local populations. Additionally, variation in the initial frequency of pesticide resistance genes and intensity of selection force by pesticide application may result in different pesticide resistance development rates among local pest populations [97], which may need to be considered to determine the velocity of acaricide resistance development and prevalence [19, 98–100].

As described above, *T. urticae* populations inhabiting stable woody plant habitats (fruit trees and roses) were geographically differentiated into genetically structured groups in Japan, but such differentiation did not occur in populations inhabiting annual herbaceous crops [69]. Goka [101] assessed resistance to two acaricides, pyridaben and fenpyroximate (used in Japan since 1991), in *T. urticae* populations collected from 48 deciduous fruit tree orchards and rose gardens and from 42 patches of herbaceous plants (some of which had been grown in greenhouses) from spring 1993 to autumn 1994. They found a rapid development of acaricide resistance in the woody plant populations. Only 7 and 8 of 48 populations showed 100% mortality after the application of pyridaben ($200 \, mg \, l^{-1}$) and fenpyroximate ($50 \, mg \, l^{-1}$), respectively. In contrast, resistant individuals were found in more than 80% of the populations; more than half of the tested individuals were alive in 24 and 10 populations after the application of pyridaben and fenpyroximate, respectively. Remarkably, no individual died by either acaricide in six populations (Figures 2(a) and 2(b)). In contrast, resistant individuals were found in a few populations after application of the acaricides, and more than 50% of individuals survived in an identical population (Figures 2(c) and 2(d); arrows). Different velocities of resistance development between these habitats were not caused by differences in the frequency of acaricide application (Figure 3). Instead, these phenomena may have occurred due to differences in mite population size which can be harbored in the habitat and habitat stability. Mite populations on woody plants are stable residents such that gene flow between them results in the strong likelihood

Woody plants (fruit trees and roses)

(a) Pyridaben (200 mg L^{-1})

Woody plants (fruit trees and roses)

(b) Fenpyroximate (50 mg L^{-1})

Herbaceous crops

(c) Pyridaben (200 mg L^{-1})
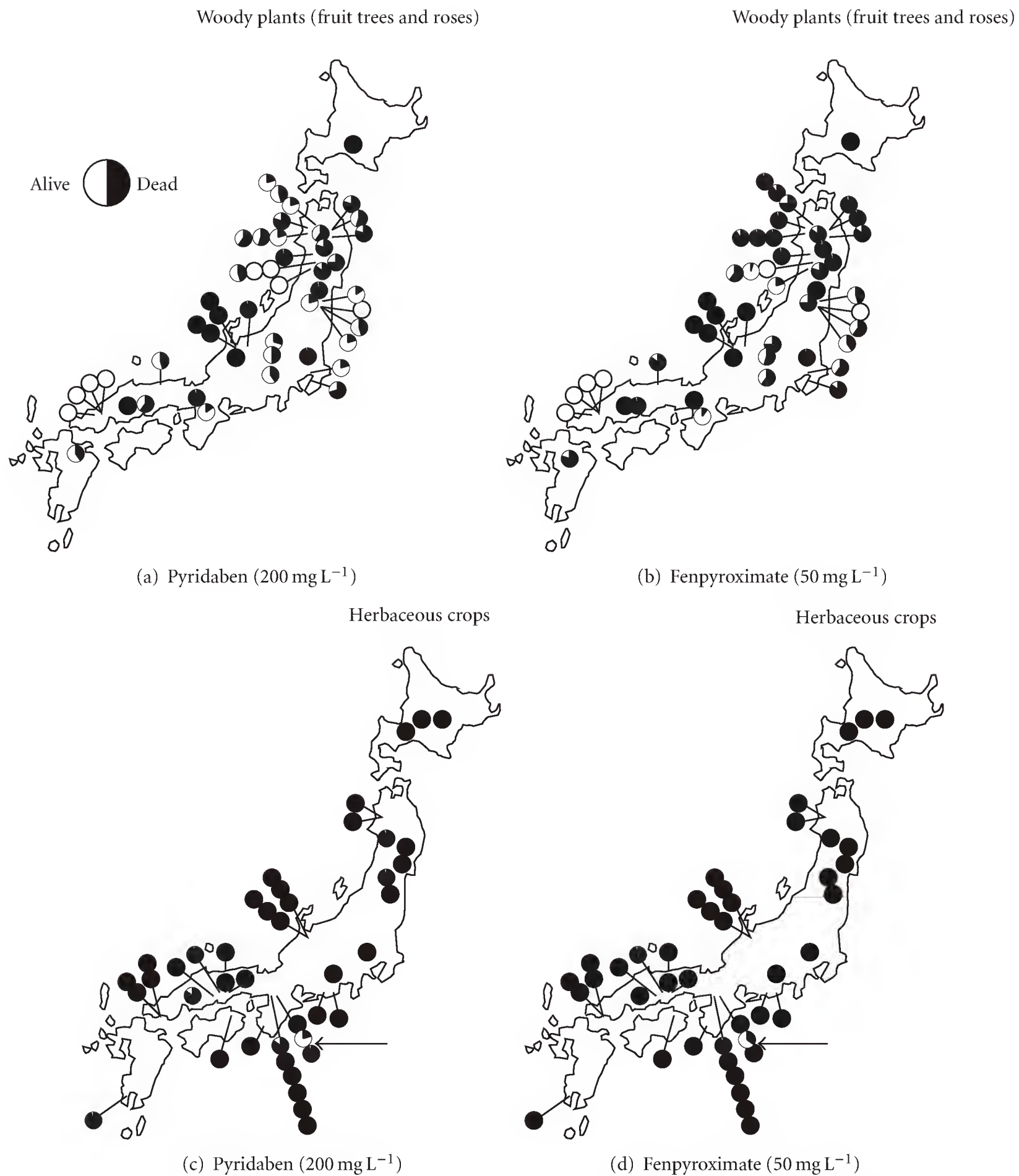
Herbaceous crops

(d) Fenpyroximate (50 mg L^{-1})

FIGURE 2: Geographic variation of susceptibility to acaricides in 48 and 42 *T. urticae* populations collected from woody plants (fruit trees and rose plants) (a), (b) and herbaceous crops (c), (d).

of selection and accumulation of rare genes for resistance, whereas those on herbaceous crops are unstable transients such that rare genes would be unlikely to be retained for long periods [101]. Yet, resistance to the acaricides was obviously developed in a single population on herbaceous plants (Figure 3; arrows), which could have resulted from founder effects.

In the assessment by Osakabe et al. [89], *P. citri* showed higher genetic diversity in acaricide resistance genes within a grove and within a tree than between groves; a two-level nested analysis of variance revealed significant difference in fenpyroximate resistance among trees within groves ($P < .025$) but not among groves ($P > .25$). This may reflect the population structure revealed using gene markers, as described above. Similar trends also occurred in the distribution of etoxazole resistance [89]. A variation in fenpyroximate susceptibility was found even among patches within a tree. In a field study by Yamamoto et al. [102], strong spatial aggregation of the hexythiazox resistance gene in *P. citri* was identified both between trees and within a single tree in a citrus grove. This result suggests that acaricide resistance genes are spread based on the population
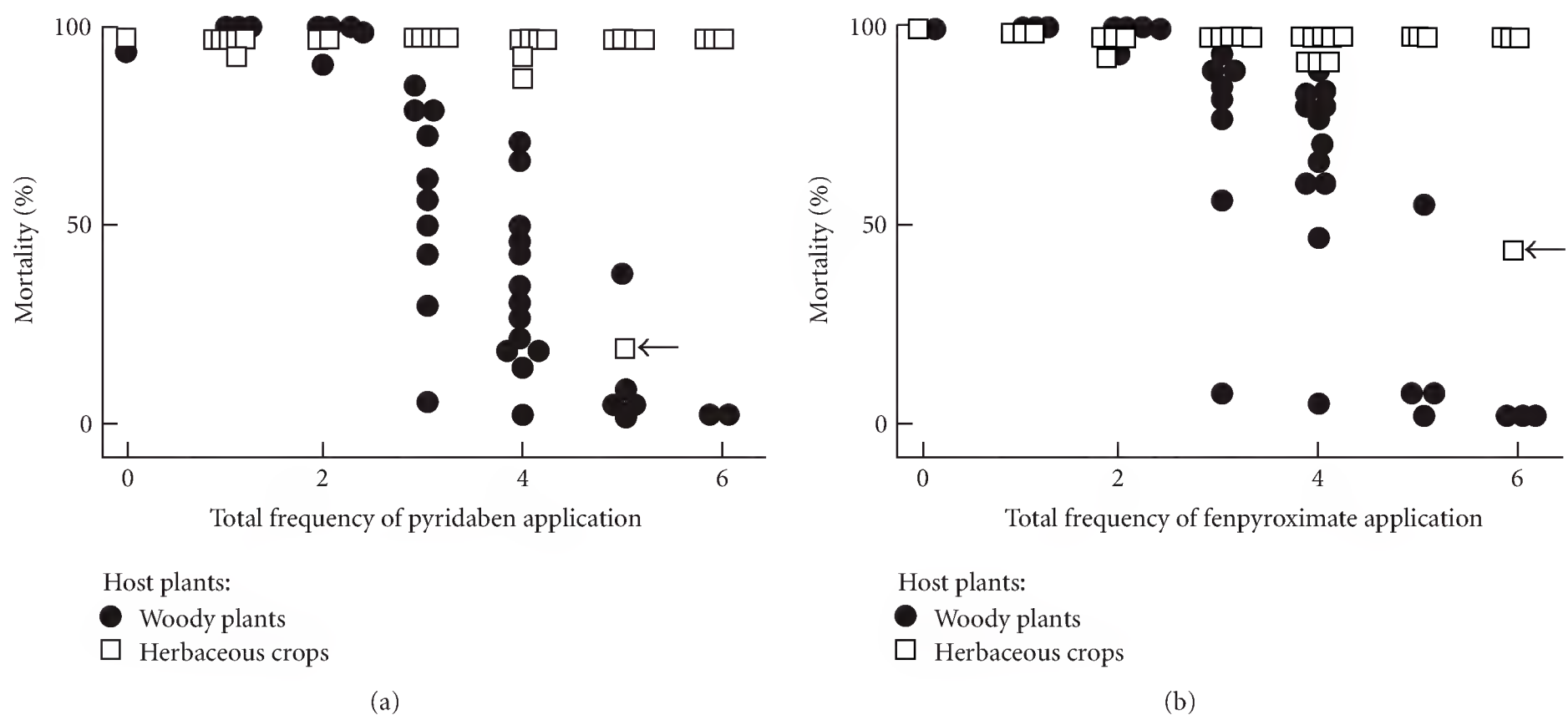
(a)   (b)

Figure 3: Correlation between total field applications of acaricides ($200\,\mathrm{mg\,L^{-1}}$ pyridaben; $50\,\mathrm{mg\,L^{-1}}$ fenpyroximate) and mortality for local populations.

structure, that is, limited crawling dispersal within foliage [57] resulting in between-patch diversity and frequent longer aerial dispersal by ballooning resulting in between-grove homogeneity.

A study of genetic differentiation of a *T. urticae* population on roses in a greenhouse estimated short dispersal distances and thus low spread velocity of acaricide resistance genes [74]. Nevertheless, milbemectin resistance genes, which had been distributed only partially in the population in July 2006, spread over the bed after only 2 months (Uesugi et al. unpublished data). During those 2 months, the roses were sprayed twice with milbemectin. The mechanism of such quick resistance development is unclear. Given that gene flow among patches within a bed did not occur frequently, an increase in the gene frequencies as effects of selection likely functioned to develop resistance. As Navajas et al. [40] noted, human-promoted passive transportation through agricultural practices might facilitate the quick spread of resistance genes.

7. Comprehensive Aspects of Acaricide Resistance Evolution

The initial frequency of resistance genes and the gene dominance are influential factors that determine the rate of resistance development [44]. In general, given a nonstructured population, mutated alleles associated with pesticide resistance likely exist at very low frequencies, and so in most of the resistance alleles are expected to be heterozygotes. Nevertheless, theoretical model experiments have revealed that in a metapopulation condition even lower-frequency alleles form concentrated foci in a set of subpopulations with certain qualifications for selection of pesticides, extinction

of subpopulations, and migration [44]. With these qualifications, subpopulation extinction and subsequent migration and reconstruction possibly occur based on incidental changes in gene frequency through founder effects. This sometimes results in the construction of subpopulations in which the resistance allele frequencies are relatively higher, making selection by pesticides more effective for resistance development.

Data concerning genetic structure suggest that spider mite populations are metapopulations. Although Uesugi et al. [87] did not find differentiation within an apple orchard, there was significant heterozygote deficiency (significantly positive F_{IS} value) in all trees, suggesting that Wahlund effects may have been caused by the division of the population into small breeding patches. Young gravid females are the main dispersers of spider mites when they experience high density [83]. Whether they disperse aerially or crawl, the dispersers may arrive alone on a new feeding site. These dispersal behaviors and restricted dispersal ability by crawling imply that founder effects frequently act on spider mite colonies, promoting the development of resistance foci through selection for acaricides. The resistance genes will then spread limitedly to neighboring colonies by gene flow or colony expansion by propagation. Moreover, the resistance genes may be spread from foci to other distant habitats by aerial dispersal in each species.

We have no data about the probability of new-linkage formation among resistance genes controlled by distinctive loci on the same chromosome by recombination in field populations. The frequent occurrence of resistance foci may increase the mating chances between mites bearing different resistance genes and might be followed by the occurrence of hitchhiking effects. Precise genetic experiments will be necessary to elucidate this issue.

8. Acaricide Resistance Management and Globalization

Species identification is a basic issue for biosecurity as injurious species must be identified to prevent their invasion and establishment in new areas [103–105]. Eight spider mite species including *T. urticae, T. kanzawai, P. citri*, and *P. ulmi* are serious cosmopolitan pests and are now listed as nonquarantine pests in the Plant Protection Law of Japan. Therefore, the risk of introducing exotic resistance alleles to inland areas is undoubtedly increasing. Even if there are inland populations resistant to the same acaricide, an invasion of an exotic resistance gene may be a potential risk over the long term because an introduced resistance gene would not be identical to that of inland populations. Therefore, more studies on the diversity of acaricide resistance genes should be performed using genetic marker linkage maps. Moreover, methods to control the spread of such exotic resistance genes may be important to prevent the development of more complex resistance, and mechanisms of regional dispersal and resulting gene flow may have to be addressed.

9. Perspectives

Identifying a mechanism for the development of pesticide resistance is important for advancing pesticide resistance management for arthropod pests. In spider mites, past genetic and ecological studies have comprehensively suggested that the local concentration of resistance genes (increasing gene frequency in breeding patches) resulting from genetic diversity within habitats based on their biological traits and selection by acaricides and gene flow from selection sites to surroundings (local and/or regional spread of resistance) are the processes of acaricide resistance evolution.

Our study has revealed the consequences of habitat stability to acaricide resistance development [101] and implies that disturbing the expansion of incipient colonies is effective for restraining acaricide resistance development and avoiding complications with spider mites. Although natural enemies such as phytoseiid mites (Acari: Phytoseiidae) play the role of the disturber, their settlement and predation activity are important. We expect the development of new technologies for the management of natural enemies, such as artificial diet provisions [106]. Other factors possibly disturbing incipient spider mite colonies such as ultraviolet rays, which are lethal to spider mites [107], should also be exploited. Overall, the establishment of an integrated mite management strategy aimed at lowering spider mite population density may be the best way to manage acaricide resistance.

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