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Research Article

A Modular Cage System Design for Continuous Medium to Large Scale *In Vivo* Rearing of Predatory Mites (Acari: Phytoseiidae)

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A new stackable modular system was developed for continuous *in vivo* production of phytoseiid mites. The system consists of cage units that are filled with lima beans, *Phaseolus lunatus*, or red beans, *P. vulgaris*, leaves infested with high levels of the two-spotted spider mites, *Tetranychus urticae*. The cage units connect with each other through a connection cup, which also serves for monitoring and collection. Predatory mites migrate upwards to new cage units as prey is depleted. The system was evaluated for production of *Phytoseiulus persimilis*. During a 6-month experimental period, 20,894.9 ± 10,482.5 (mean ± standard deviation) predators were produced per week. The production consisted of 4.1 ± 4.6% nymphs and 95.9 ± 4.6% adults. A mean of 554.5 ± 59.8 predatory mites were collected per harvested cage and the mean interval length between harvests was 6.57 ± 6.76 days. The potential for commercial and experimental applications is discussed.

1. Introduction

Phytoseiid mites are very effective predators used mainly in biological control of spider mites, *Tetranychus urticae* (Koch); however, phytoseiids are known to provide effective control of other mite species and some insects like thrips and white flies [1]. Zhang [2] reported that at least 20 species of phytoseiids have been made commercially available and have been applied mainly on greenhouse plants. The phytoseiid that has been most widely mass-produced and sold commercially is *Phytoseiulus persimilis* Athias-Henriot. *Phytoseiulus persimilis* is an effective biological control agent of spider mites on vegetables in glasshouses [3–5] and growers around the world use *P. persimilis* to control *T. urticae* and other tetranychid mites on crops grown in greenhouses and in the field [6, 7]. Other phytoseiid species produced commercially and used in augmentative biological control of greenhouse pests include *Neoseiulus cucumeris* (Oudemans), *N. barkeri* Hughes, *N. californicus* (McGregor), *N. fallacis* (German), *Iphiseius degenerans* (Berlese), and *Galendromus occidentalis* (Nesbitt) [2].

Current methods of mass production of phytoseiid mites such as *P. persimilis* rely on greenhouse growth of bean plants for spider mite production and later inoculation with the predatory mite. A pure spider mite culture, free of predators, is also required for rearing. Infested leaves from the pure culture are used to infest bean plants in a different greenhouse. A series of greenhouse benches are inoculated at weekly intervals to provide continuous supply of prey. Predators are later introduced to bean plants heavily infested with spider mites and grown for 2-3 weeks. A section of the bench is harvested when it has reached the maximum predator density [8]. Introduction of *P. persimilis* into the infested beans requires perfect timing to allow maximum spider mite reproduction without losing the plants to the mite infestation [8]. Predator harvesting often exposes the predators to stressful conditions of starvation and many are lost to inefficient collection methods.

Enclosed rearing systems offer the potential of greater control of environmental conditions and better containment preventing excessive losses. Several methods for rearing phytoseiid mites in enclosed systems or cages by introducing

prey have been proposed consisting of dishes with a central area limited by a channel filled with machine oil or other liquids [9–11].

Theaker and Tonks [12] reared *P. persimilis* in floating plastic leads positioned by magnets in the middle of a water-filled container to prevent mites from escaping. A similar method based on a plastic foam block or sponge positioned in the middle of a tray filled with water was described by Overmeer [13] to rear several species including *P. persimilis*, *P. macropilis* (Banks), *Typhlodromus occidentalis* Nesbitt, *T. pyri* Scheuten, *Amblyseius (Neoseiulus) fallacis* (German), *A. potentillae* (German), and *N. cucumeris* (Oudemans). A barrier is formed by placing wet tissue paper around the block with one side touching the water to maintain a continuous saturation [13]. McMurtry et al. [14] describe a method of mass rearing of phytoseiid mites by washing eggs and other spider mite stages from infested leaves. The washed spider mites are then fed to predatory mites reared using the paper-lined block in a water tray method as described by Overmeer [13]. Series of these trays are stacked inside shelved wood boxes. Shih [15] developed a method to separate the prey mites (*T. urticae*) from plant leaves and an apparatus which used pneumatic pressure to dispense a mix of the prey and corn pollen to rear *Amblyseius womersleyi* Schicha using the same lined semisubmerged block method.

Fournier et al. [16] proposed a cage system for rearing *P. persimilis* consisting of series of superimposing cylinders filled with bean leaves heavily infested by spider mites. New cylinders with infested leaves are added to the top of the series to supply new prey. The cylinders at the bottom are retired as predators move into cylinders with fresh prey [16]. Another cage system was described by Overmeer [13] consisting of two cardboard ice cream containers glued together and separated by a screen. Infested leaves are placed in the lower side and predators are introduced. New leaves are placed in the upper side where predators tend to move to find new prey. The whole system is flipped over to place new leaves while removing the old material [13].

While many enclosed rearing systems have been effective to mass-produce phytoseiid mites commercially, none of the existing enclosed systems can match the production capabilities of the open greenhouse rearing methods. An increasing level of sophistication will be required to reach a comparable level of production using enclosed systems. The objective of this study was to develop and test a refined enclosed rearing system based on the Fournier et al. [16] cage series.

2. Materials and Methods

2.1. Rearing of the Prey. Spider mites, *Tetranychus urticae* Koch, were used as prey to feed the phytoseiid mites. Colonies of *T. urticae* were established from commercial stocks provided by Syngenta Bioline, Oxnard, CA, and were reared on red kidney beans, *Phaseolus vulgaris* L., and lima beans, *Phaseolus lunatus* L., cultivars Fordhook 242 and Henderson in a greenhouse. The greenhouse was divided into two areas by using a clear polyethylene curtain. Lima beans were grown in one-half of the greenhouse using 60 × 20 × 20 cm polyethylene planters. The bottom of each planter was lined with 2.5 L perlite (Coarse, Sunshine, SunGro Horticulture, Bellevue, WA) to support and maintain humidity. A mixture of 2 : 1 potting soil (Moist control, Miracle-Gro Marysville, OH), vermiculite (Coarse, Sunshine, SunGro Horticulture, Bellevue, WA), and 5 g slow release fertilizer (N : P : K = 14 : 14 : 14) (vegetable and bedding, Osmocote, Marysville, OH) was mixed and then combined with an equal volume of a mixture of 20 g TeraGel (T-400, The Terawet Corporation, San Diego, CA), 0.5 g water soluble fertilizer (N : P : K = 24 : 8 : 16) (All-purpose plant food, Miracle-Gro, Marysville, OH), and 2.5 L tap water. The aqueous solution was allowed to equilibrate for 24 h until the water was fully absorbed by the TeraGel crystals and then it was homogeneously incorporated into the potting soil and vermiculite mixture using a gardening trowel. Seventy seeds were planted and kept for 10 days in each planter for germination. Ten days after germination, planters with young bean plants were transferred to the second half of the divided greenhouse and plants were then massively infested with *T. urticae* by placing leaves from heavily infested plants on top of them.

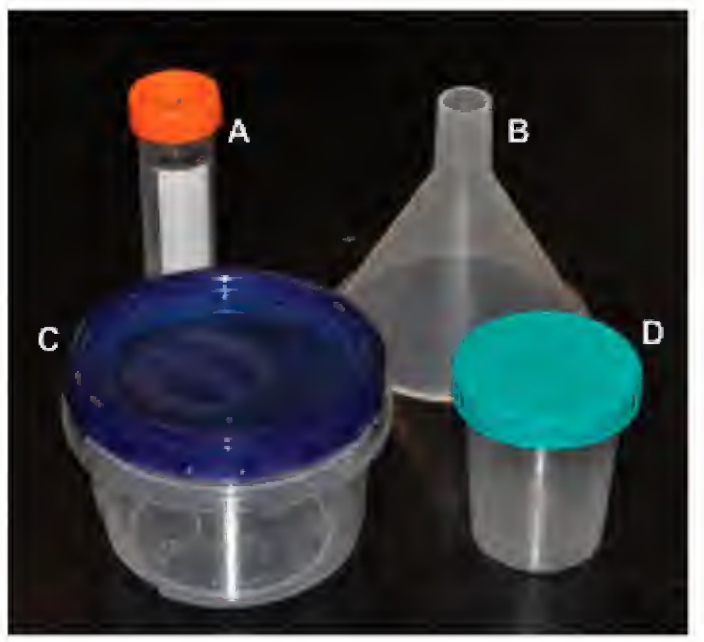
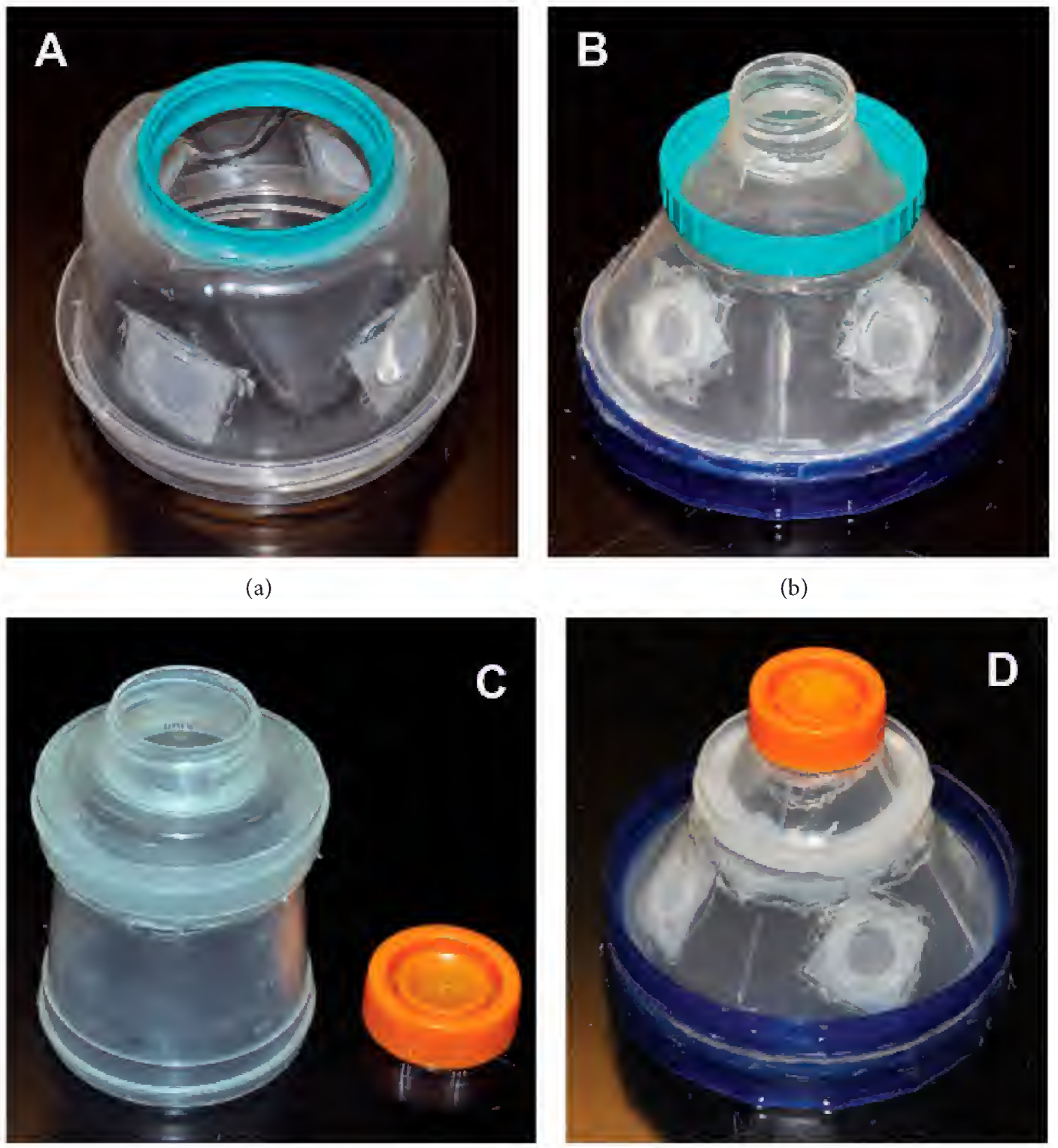


Figure 1: Materials used to construct the cage system. (A) Polypropylene 50 mL centrifuge tube with screwed cover, (B) polypropylene 250 mL lab funnel, (C) polypropylene Ziploc storage containers, and (D) high density polyethylene 120 mL specimen containers.

Spider mite infestation levels were allowed to increase for 5 days after their introduction. Fully infested plants were monitored daily to determine optimal infestation levels. Extreme infestation levels kill the bean plants inducing massive migration of spider mites. Infested bean leaves were collected when they were still alive (leaves still green) and sustain a high density of spider mites. Infested leaves were collected daily by cutting them manually using garden scissors and placing them in plastic boxes. Boxes with infested bean leaves were stored at 15°C for 1 to 7 days.

2.2. Predatory Mite Rearing and Cage Design. Although the rearing system presented herein is suitable for any phytoseiid predator of spider mites, *P. persimilis* was used as the basis to test the system. The rearing system is based on the same principles of Fournier et al.'s [16] stacked cage method, but



(a) (b)

(c) (d)

FIGURE 2: Basic cage system modular components. (a) Cage bottom, (b) cage cover, (c) connection cup, and (d) cage series stand.

we designed a unique modular structure of identical cage units. Stackable modular units were constructed from 473 mL Ziploc storage containers (Ziploc Twist'n Loc, S.C. Johnson & Son, Inc., Racine, WI), 250 mL plastic laboratory funnels (Fisherbrand 10-500-2, 10.5 cm dia. × 10.3 cm H), plastic 50 mL centrifuge tubes (Corning No. 430897), and 120 mL specimen containers (LSS number 9BC-135972) (Figure 1). Materials for cage construction were chosen based on the quality of a water-tight screwed cover closure. Snap closures tend to fail with continuous use and mites quickly find escape openings. The cage system consisted of 4 basic parts that were modified to fit together: a cage bottom (Figure 2(a)), a cage cover with funnel connection (Figure 2(b)), a connection cup (Figure 2(c)), and a multiuse funnel to serve as stand (Figure 2(d)). The covers of the Ziploc containers (Figure 1(c)) were cut to allow the insertion of the lab funnels to the cage covers (Figure 1(b)). The tips (narrow ends) of the funnels were cut to install "male" screw sections of centrifuge tubes to allow closure when required (Figures 2(b) and 2(d)). "Female" screw sections of the covers of specimen containers (Figure 1(d)) were cut and glued to the funnels and bottoms of the cage to allow connection with other cage units (Figures 2(a) and 2(b)). "Male" screw sections of centrifuge plastic tubes (Figure 1(a)) were also glued to the bottom of the connection cups (Figure 2(c)) to allow closure during mite collection and movement when connected to new cage units. Connection cups were also fitted with a second "male" screw section in the bottom allowing connection to both ends (Figure 3(c)). A cage unit consisted of bottom, top, and connection cup (Figure 3(a)). The system stand was used only in the starting cage (Figure 3(b)) and was fitted with a "male" instead of a "female" screw section from specimen containers (Figure 2(d)) to allow connection to the bottoms (Figure 2(a)) of the cages. Four circular windows (22 mm dia.) were cut on the sides of the cages bottom and four more (17 mm dia.) were cut on the sides of the funnels for ventilation. Only one circular window (10 mm dia.) was cut on one side of the connection cups to reduce excessive loss of moisture. Nylon screen 85 μm mesh (Small Parts Inc., U-CMN-85) was used to seal the windows preventing mites

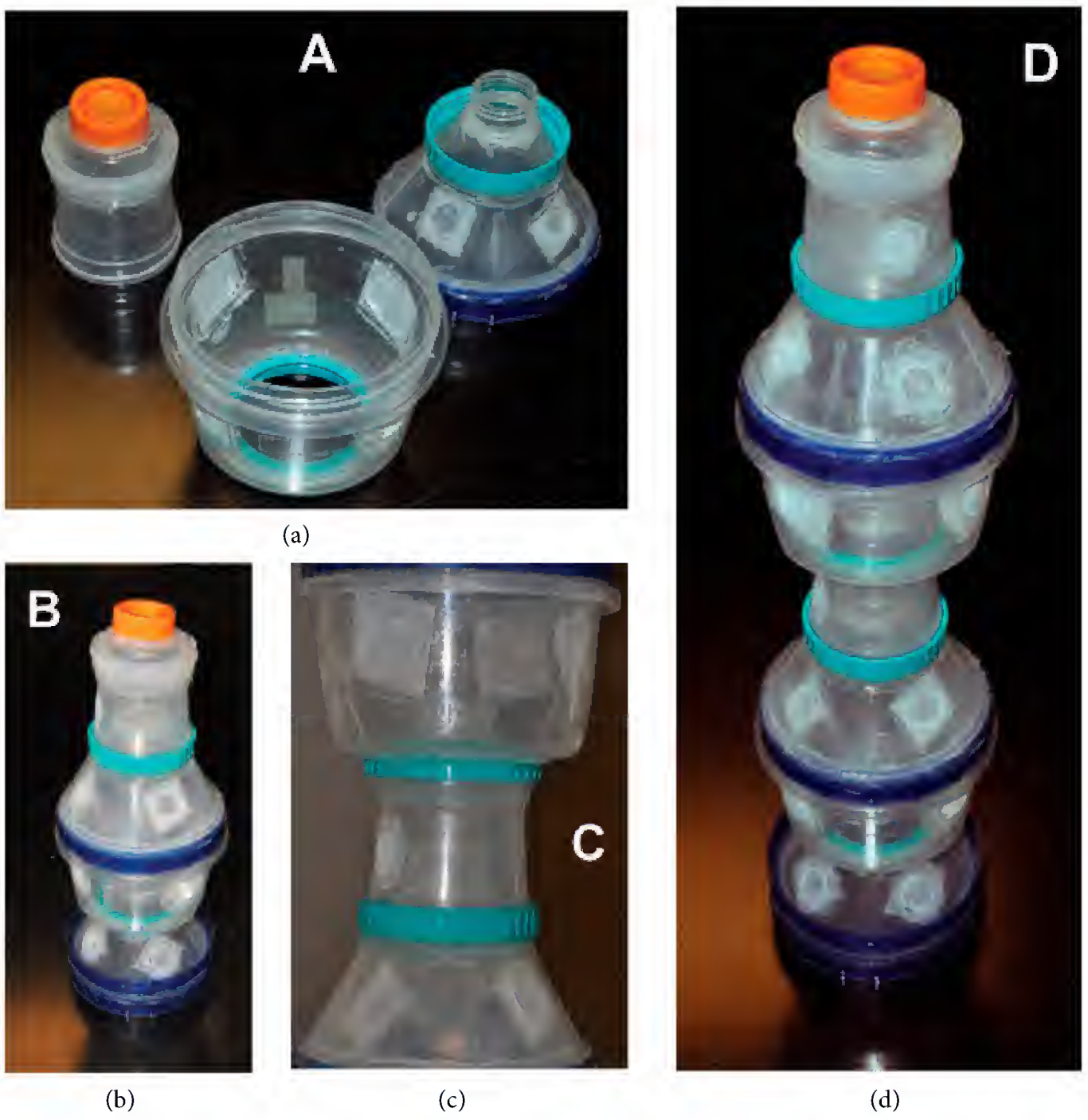


FIGURE 3: Cage system assembly. (a) Cage unit components, (b) cage unit assembled, (c) connection cup with second cage unit fitted, and (d) cage series assembly of two cage units.

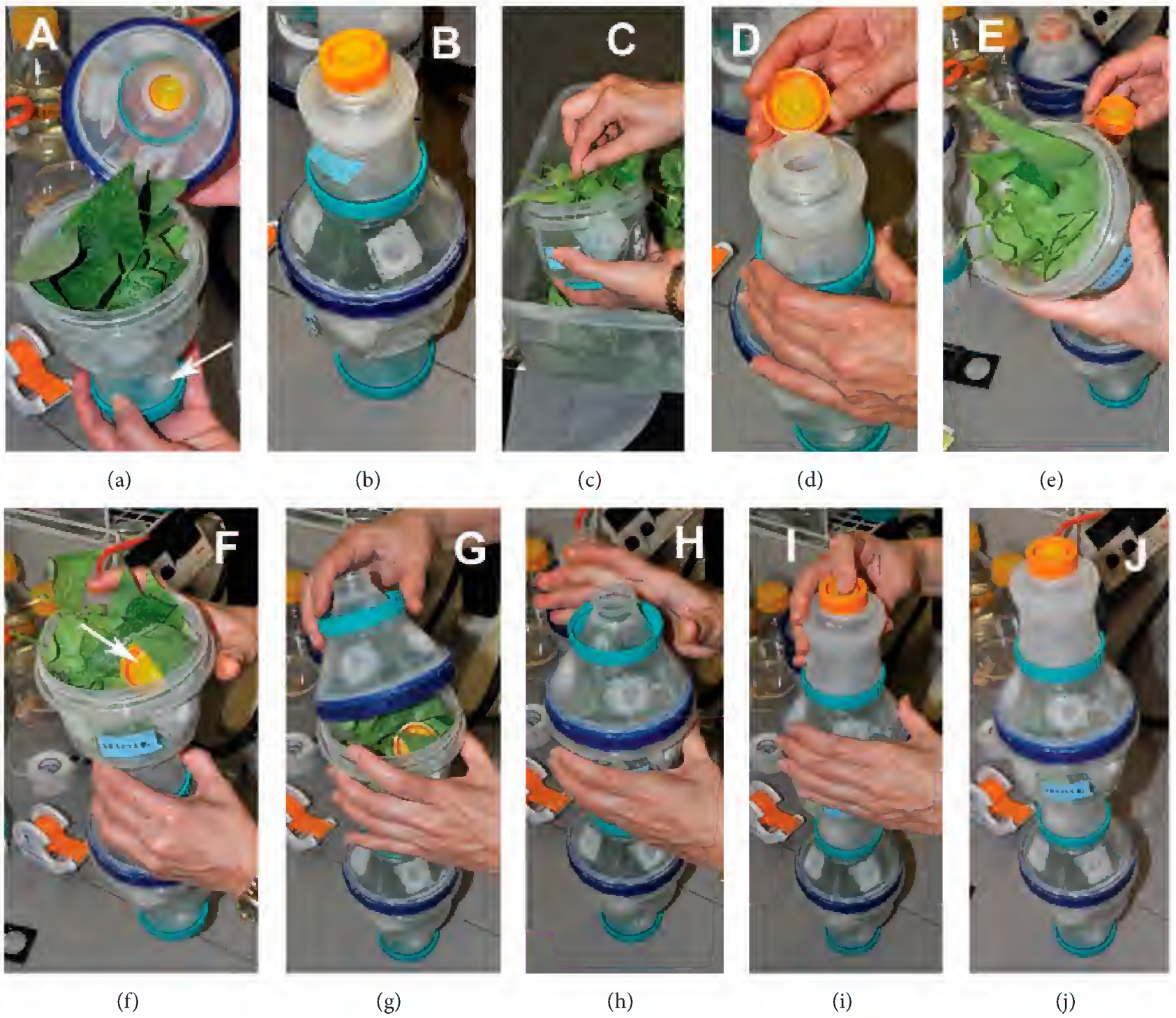
from escaping. Cage units were designed to fit together in a modular way by connecting the bottom to the connection cup (Figures 3(c) and 3(d)).

Bean leaves heavily infested with *T. urticae* were placed inside each cage unit stacked vertically to allow mites to move up (Figures 4(a) and 4(c)). A cage series can be started by introducing a few adult predatory mites (10–100) into a cage unit newly filled with infested bean leaves. To start a cage series, a connection cup with mites is fitted to the bottom of a new cage unit (Figure 4(a) arrow and Figure 4(b)). This cup can later be replaced by the stand described in Figure 2(d) to provide better stability.

After prey mites had been depleted, a new unit is attached to the top of the old unit by removing the cover of the connection cup (Figure 4(d)) to allow predators to move into the new unit. The cover (full of predators) is placed inside the new unit (Figures 4(e) and 4(f)) and the cage is closed with a new funnel (Figures 4(g) and 4(h)). A new connection cup is attached to the top of the funnel (Figure 4(i)) to complete the system (Figure 4(j)). Nymph and adult predators tend to migrate to the upper end of the cage series and accumulate in the uppermost connection cup feeding on migrating spider mites.

2.3. Evaluation. The cage system was evaluated using *P. persimilis* as model. Evaluation started on 1 January 2007 by establishing 21 cage series. Each cage series started with approximately 100 adult predators. New cage series were created using predatory mites produced by the initial series. Some cage series had to be terminated and replaced due to contamination by other predatory mite species (*Neoseiulus* sp.) from the greenhouse spider mite production. Series increased in number to a maximum of 48 at the end of the study on 1 July 2007. The study was conducted in an environmentally controlled room at 26 ± 1°C, 80 ± 5% RH, 14 h photophase, and 10 h scotophase.

Cage units were added to the top of each cage series as described above. Prey mites consisting of *T. urticae* were reared as described above using *P. lunatus* Henderson variety. When the connection cup contained a visibly high density of predatory mites, the cup was quickly disconnected from the



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

(f) (g) (h) (i) (j)

FIGURE 4: Cage system operation. (a) A cage series starts with a connection cup with predators connected to a cage bottom filed with spider mite-infested bean leaves. (b) The starting cage unit is closed and a connection cup is fitted. (c) When spider mites have been depleted by the predators, a new cage unit is prepared. (d) The cover of the connection cup is removed to allow predators to move to the new cage. (e) A new cage bottom is fitted to the connection cup. (f) The connection cup cover is placed inside the new cage. (g) The new cage is covered and (h) sealed, and (i) a new connection cup is fitted to the cover. (j) The process can be repeated by adding a third cage unit when prey has been depleted. At this point, the bottom connection cup can be replaced by the stand piece.

series, inverted, taped to make predator fall to the bottom, and filled with 70% ethanol to kill and preserve the predatory mites. Mites were counted and the numbers were recorded. Data consisting of days between harvest, collection date, cage series, and number of *P. persimilis* collected were recorded. Data were analyzed using single-variable statistics to determine means of mite production per week, mites produced per cage, and mean time from initiation to collection.

In this study, predatory mites were collected and killed with 70% ethanol in order to obtain precise numbers. However, live predators can be quantified while alive using less precise methods. One method is based on weight: first, a mean of individual weight is determined by weighing groups of mites in a precision balance; second, an empty collection cup (with cover) is weighed and used to collect predatory mites by attaching it to the top of the cage system. The cup full of mites can be closed (with the previously weighed cover) and weighed a second time. The weight of the live mites can be determined by subtracting the weight of the empty cup from the weight of the full cup. Another method consists of determining the number of mites fitting in a given volume. Mites can be forced by gentle vacuum into receptacles with known volume. When filled, the receptacle can be emptied by reversing the airflow.

3. Results and Discussion

During the six-month evaluation period, the mean weekly production was 20, 894.9 ± 10, 482.5 (mean ± standard deviation) *P. persimilis*. Production consisted of 4.1 ± 4.6% deutonymphs and 95.9 ± 4.6% adults. Overall production mean was 554.5±59.8 mites per harvested cage and a mean of 36.7± 17.0 cages were harvested per week. The mean interval of time between harvests was 6.57 ± 6.76 days; however, the length of the harvest intervals did not have a normal distribution and the median was 4 days and the 75% quartile

was 5 days (Figure 6). The top quartile consisted of intervals ranging from 6 to 62 days in length and the 90% percentile was 14 days. Based on this analysis, the time interval between harvests should not exceed 14 days. If by 14 days the population of predators is still too low to justify harvest, the cage series should be terminated and a new series should be generated from predator production of a healthy series.

The connection cup usually contains large quantities of spider mites, which constantly migrate when they are in high densities. As predatory mites increase in numbers, they consume all the prey in the connection cup. Juvenile predatory mites (young adults and deutonymphs) tend to rapidly migrate upwards to the new cage unit. Gravid adult females usually remain in the bottom cage unit until they oviposit. As the last eggs are oviposited and prey is depleted, females move upwards to the new cage unit. As the population of predatory mites increases, it becomes necessary to add new cage units within increasingly shorter periods of time. Empty cage units at the bottom can be removed after all the eggs have hatched and the juveniles have moved to new cage units. The connection cup at the top of the series serves as an indicator of predatory mite population. The predators in the connection cup may be recycled by fitting a new cage unit or harvested by removing the connection cup and closing it with an unmodified cover of a specimen container. Decision to harvest the predatory mites depends on the density of juveniles in the connection cup (Figure 5). Once the predatory mite population is well established in a cage series, it becomes necessary to harvest the predatory mites every 2 to 4 days depending on the quality of prey provided. Harvested mites can be used to start new cage series, for field releases, or for use in experiments. A cage series can be continuously producing predatory mites indefinitely as long as new cage units are added to the top of the series.

Peak weekly production occurred during the week between March 11 and 18 with a total of 38,097 adults and nymphs harvested (Figure 7(a)). During this period, 41 cage series were in operation (Figure 7(b)). Mean production per harvested cage was more or less consistent during the experimental period (Figure 7(c)). The total weekly production dropped sharply by the end of the experiment even as the number of series increased; however, the number of mites per harvested cage was increasingly consistent evidenced by the decrease of the standard deviation (Figure 7(c)).

A study of three-trophic-level impact of secondary chemicals present in lima beans showed that high levels of linamarin present in Henderson lima beans tend to accumulate in the predatory mites after several generations [17]. Accumulation of linamarin may have been the reason the production dropped by the end of the study even as the number of cage series increased. The use of this variety is not recommended for continuous production of phytoseiid mites. Fordhook 242 lima beans provide a better alternative because they contain only trace amounts of linamarin (M. G. Rojas unpublished). Henderson lima beans were selected for this experiment because they are easy to grow and the size of their leaves is optimal to fit inside the cages described in this study. Fordhook 242 lima beans have larger leaves, which must be folded or cut to fit in the cages. Another good choice of host plant is red beans, which have similar leaf sizes to those in Henderson lima beans. However, a system could be constructed with larger cage units providing more space to accommodate larger leaves.

The size limits for the system have not been determined, but 8 liter (2-gallon) size units have been constructed and tested successfully (Figure 8). In theory, the system can be scaled up to accommodate production levels of millions of mites per week. Potential size limits include the structural integrity of currently available materials taking into account the weight of the leaves that must be held by the cage units. The tightness of the closures between cage unit connections can be more difficult as cage size increases. The tolerance of closures between cage units cannot exceed 100 μm to contain

Figure 5: Connection cup with *P. persimilis* after prey had been depleted. A piece of paper can be introduced to the connection cup to increase the surface area.

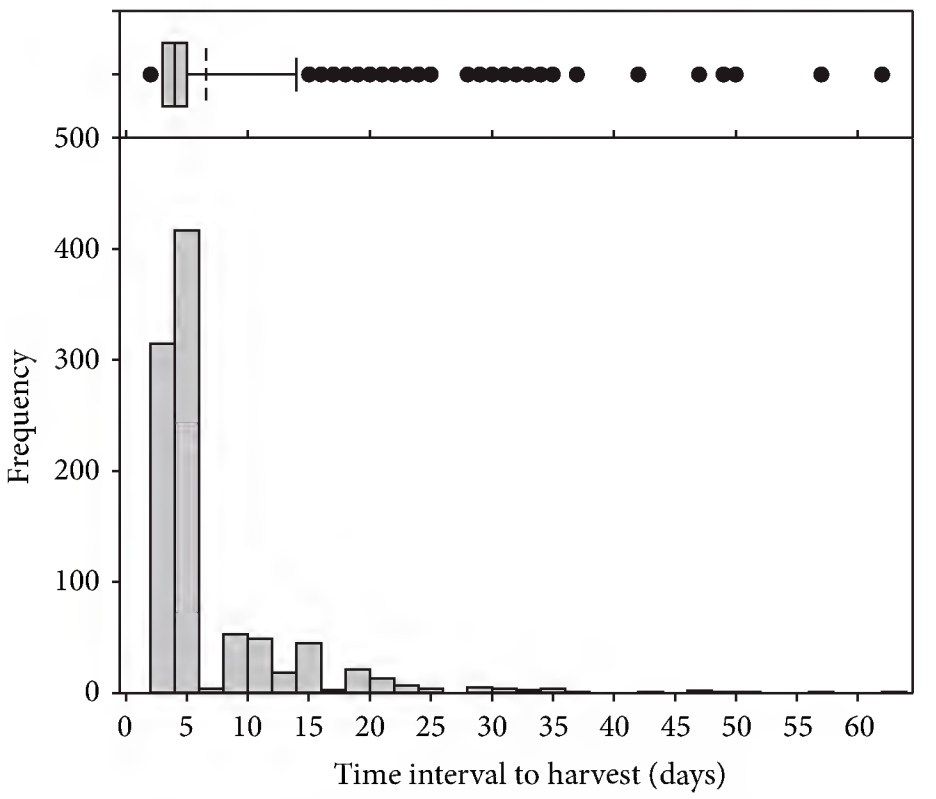


Figure 6: Frequency distribution of predator harvest intervals. Box plot (top): bars represent 25% and 75% quartiles, line between bars represents the median, dashed line represents the mean, bracket represents the 90% percentile, and dots represent outliers. Bar plot (bottom): bars represent 2-unit classes.

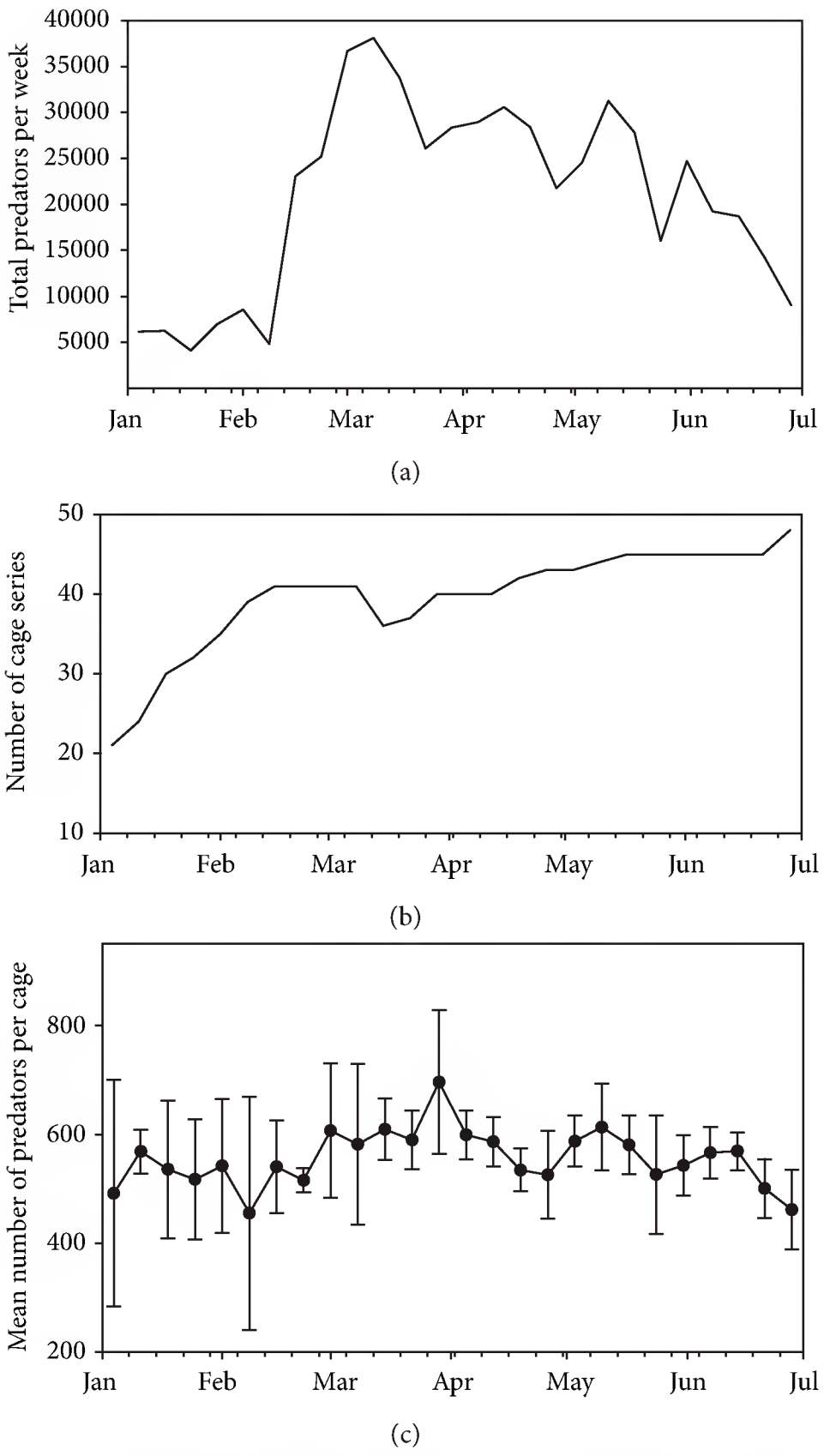


FIGURE 7: Production of *P. persimilis*. (a) Total weekly production. (b) Number of cage units in production. (c) Means of predators produced per harvested cage; brackets represent standard deviation.

the mites within the cages, and this becomes increasingly difficult as the size of the screw cups increases.

4. Conclusions

The modular cage system presented in this study has been shown to be a consistent and robust method to produce phytoseiid mites. The system is particularly suitable for medium to large scale rearing of *P. persimilis*. This system provides a good alternative for phytoseiid mites rearing and potentially can be scaled up for mass production.

Conflict of Interests

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

FIGURE 8: Larger size cage system design.

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Research Article

Ovicidal Activity of *Couroupita guianensis* (Aubl.) against *Spodoptera litura* (Fab.)

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Hexane, chloroform, and ethyl acetate extracts of *Couroupita guianensis* leaves were studied for ovicidal activity against *S. litura*. All the extracts showed ovicidal activity against *S. litura*. Maximum activity was noticed in hexane extract and it showed the least LC_{50} and LC_{90} values; the regression equation was also higher than the other extracts. All the analyzed values showed homogeneity variance. The active hexane extract was fractionated and eight fractions were isolated. The fractions were studied at different concentrations. Among the fractions, fraction 8 showed maximum ovicidal activity with least LC_{50} and LC_{90} values. Fraction 8 differed statistically from the other fractions; the regression equation value was higher than the other fractions. All the *P* values obtained from regression analysis were significant. The results of the present investigation clearly suggest that the active fraction could be purified to isolate active compound(s) and could be used to develop an insecticidal formulation to control economically important agricultural pests.

1. Introduction

India is an agricultural country and more than 80% of the population depend on agriculture [1]. Pathogenic organisms and insect pests cause crop loss of 120 billion US dollars worldwide and reduce the yield by 20–40% [2]. In India, approximately 18% of food grains are lost due to pathogens and insect pests. To control the pests and reduce the loss, different chemical pesticides are used. Application of chemical pesticides is polluting the environment, causing ill effects on nontarget organisms, developing resistance, and causing resurgence of pests [3]. These call for an alternative to chemical pesticides through natural means of pest control, including vigorous search for new sources of botanical insecticides [4]. Plant-based pesticides are highly suitable since they have low toxicity, are easily biodegradable, and have multimode of action [5]; they are suitable for organic agriculture [6].

Botanical extracts are used as insecticides for centuries and their active compounds reduce the opportunity for the development of insect resistance [7]. Plants have evolved a range of adaptations to increase their survival and reproduction by minimising the impact of phytophagous insects. Plants defend themselves from herbivores with the help of secondary metabolites produced by them and these secondary chemicals can act as repellents or toxins to herbivores and affect their behaviour, growth, or survival. Volatile plant signals attract natural enemies of the herbivore insect pests [8]. Presently, botanicals are used as insecticides which constitute only 1% of the world insecticide market [9].

Plant-derived substances have multimode of actions against different agricultural pests and act as antifeedants [10] and larvicidal [1] agents; they reduce adult emergence and increase adult abnormalities [11, 12]; they inhibit larval growth [13] and cause ovicidal and oviposition deterrent activities [14]; and they bring about cytological changes [5].

*Couroupita guianens*is leaves extracts showed antifeedant, larvicidal, and ovicidal activities against *Helicoverpa armigera* [15, 16] and antifeedant activity against *Spodoptera litura* [17]. *S. litura* is a major polyphagous pest attacking more than 150 host species affecting the yield [18]. It causes serious damage to young plants and the buds of different vegetable crops in Thiruvallur and Kancheepuram districts of Tamil Nadu. The present study was aimed to evaluate the ovicidal activity of

different crude extracts and fractions of *C. guianensis* against *S. litura*.

2. Materials and Methods

2.1. Plant Collection. Leaves of *C. guianensis* were collected from Loyola College Campus, Chennai, Tamil Nadu, India. The plant was identified by Dr. M. Ayyanar, Taxonomist, Entomology Research Institute, Loyola College. The voucher specimen (ERIH: 1310) was deposited at the institute herbarium. The plant material was shade-dried at room temperature and powdered coarsely. The plant materials were sequentially extracted using hexane, chloroform, and ethyl acetate. The active hexane extract was fractionated using silica gel column chromatography with increasing polarity of hexane : ethyl acetate combinations. Isolated fractions were concentrated using vacuum rotary evaporator with reduced pressure and the collected fractions were stored at 4°C in the refrigerator [15].

2.2. Insect Culture. Egg masses of *S. litura* were collected from groundnut field at Tiruttani in Thiruvallur District of Tamil Nadu. The eggs were surface-sterilized with 0.02% sodium hypochlorite solution, dried, and allowed to hatch. After hatching, the neonate larvae were reared on leaves of castor, *Ricinus communis*, till prepupal stage. Sterilized soil was provided for pupation at room temperature (27 ± 2°C) with a photoperiod of 14 : 10 (light : dark) and 75 ± 5% relative humidity in insectary. After pupation, the pupae were collected from the soil and placed inside the oviposition chamber. After adult emergence, cotton soaked with 10% (w/v) sugar solution with few drops of multivitamins was provided for adult feeding to increase the fecundity. Potted groundnut plant was kept inside adult emergence cage for egg laying. After hatching, the larvae were fed with tender castor leaves. The eggs laid by the laboratory reared insects were used for the present study [10].

2.3. Ovicidal Activity. The ovicidal activity of the crude extracts and fractions was studied by spraying them on freshly laid eggs of *S. litura*. The sprayed concentrations were 5, 10, 25 and 50 mg/mL for crude extracts and 125, 250, 500 and 1000 μg/mL for fractions. Spray solution of 0.5 mL was used per replicate. Azadirachtin was used as positive control [19]. Five replicates were maintained for each treatment with 20 eggs per replicate (total $n = 100$). The experiment was conducted at laboratory conditions (room temperature of 27 ± 2°C with 14 : 10 (light : dark) photoperiod and 75 ± 5% relative humidity). The number of eggs hatched in control and treatments was recorded up to 96 hrs. Percent of egg mortality was calculated according to Abbott [20].

2.4. Statistical Analysis. The ovicidal activity was analysed using one-way ANOVA. Significant differences between treatments were determined using Tukey's multiple-range HSD tests ($P \leq 0.05$). Analyses were performed with the original data after transformation with various approaches (the arcsin, logarithmic, and square root methods). The distribution of the fraction data did not show significant deviations from normality. Shapiro-wilk test for original crude data showed normality. Linear regression analyses were performed for all dose-response experimental data. LC_{50} and LC_{90} values were calculated using probit analysis [21].

3. Result

Ovicidal activity of different crude extracts of *C. guianensis* against *S. litura* is presented in Table 1. Maximum ovicidal activity of 67.33% was observed in hexane extract at 50 mg/mL concentration. The chloroform and ethyl acetate extracts showed ovicidal activity of 47 and 42%, respectively. Chloroform and ethyl acetate extracts showed statistically similar activity. Hexane extract was statistically different from chloroform and ethyl acetate extracts. At 25 mg/mL concentration, hexane extract exhibited 51.17% ovicidal activity against *S. litura* followed by chloroform and ethyl acetate extracts. All the three extracts statistically differed from each other at 25 and 50 mg/mL concentrations. Hexane extract exhibited 39.52% ovicidal activity at 10 mg/mL concentration against *S. litura* which was statically similar to chloroform extract that showed 31.20% ovicidal activity (P value 0.63). Lowest concentration of hexane and chloroform extracts showed statistically similar (P value 0.92) ovicidal activity. All the concentrations of ethyl acetate extracts showed minimum ovicidal activity. The homogeneity of variance was significant at all the analyses; also the ANOVA was significant (P value 0). The R^2 indicated that increasing concentration of the extracts increased the activity (Table 1). Regression ANOVA derived from all the three extracts showed significant value (P value 0).

The minimum quantity of hexane extract needed to kill 50% eggs of *S. litura* is shown in Table 1. Ethyl acetate extract required maximum quantity (55.94 mg/mL) for 50% egg mortality of *S. litura*. The obtained χ^2 values were significant for all the tested extracts. The probit analysis clearly indicates that the hexane extract has the potential to kill the eggs of *S. litura*.

Bioassay-guided fractionation of hexane extract was done and finally 8 fractions were obtained; they were screened at different concentrations. Among the fractions tested, fraction 8 showed maximum ovicidal activity of 30.46% at 125 μg/mL concentration (Table 2) followed by fractions 3 and 7 which showed ovicidal activity of 28.24 and 23.91%, respectively. Fractions 3, 7, and 8 were statistically similar (P value 0.15). Minimum ovicidal activity of 4.32% was noticed in fraction 5. Fractions 4 and 2 were statistically similar to fraction 5 (P value 0.15). At 250 μg/mL concentration, fraction 8 exhibited 51.05% ovicidal activity. Minimum ovicidal activity was noticed in fraction 4. Fractions 1, 3, and 7 exhibited more than 30% ovicidal activity. Fraction 8 showed 59.82% ovicidal activity at 500 μg/mL concentration followed by fractions 7, 3, and 1. Maximum ovicidal activity of 71.69% was noticed in fraction 8 at 1000 μg/mL concentration followed by fraction 7 which exhibited 60.93% ovicidal activity. Minimum ovicidal activity of 19.53% was noticed in fraction 5 which was statistically similar to fraction 4 (P value 1). Fractions

Table 1: Ovicidal activity and effective concentrations (mg/mL) of *Couroupita guianensis* crude extracts against *Spodoptera litura*.

Solvent extract	Concentration (mg/mL) 5	10	25	50	R	R^2	Regression equation	P value	LC_{50}	LC_{90}	χ^2
Hexane	21.98 ± 4.03^{b}	39.52 ± 5.94^{b}	51.17 ± 5.94^{c}	67.33 ± 4.03^{b}	0.92	0.84	26.78 ± 0.899	0.000	28.05	82.50	44.04*
Chloroform	23.16 ± 5.27^{b}	31.20 ± 5.20^{b}	41.82 ± 5.52^{b}	47.57 ± 4.70^{a}	0.84	0.70	24.60 ± 0.050	0.000	49.99	145.40	31.02*
Ethyl acetate	6.93 ± 4.77^{a}	16.16 ± 4.20^{a}	24.41 ± 2.12^{a}	42.85 ± 6.26^{a}	0.95	0.89	5.76 ± 0.75	0.000	55.94	108.28	36.31*
ANOVA	Df 2, 12, F.18, 35 P 0	Df 2, 12, F.26.15 P 0	Df 2, 12, F.39.31 P 0	Df 2, 12, F.32.54 P 0							
Homogeneity	0.67	0.52	0.13	0.38							

Means followed by the same letter do not differ significantly using Tukey's test ($P \leq 0.05$) and complete regression equations; * χ^2 values are significant.

Table 2: Ovicidal activity and effective concentrations (μg/mL) of *Couroupita guianensis* hexane fractions against *Spodoptera litura*.

Fractions	Concentration (μg/mL) 125	250	500	1000	R	R^2	Regression equation	P value	LC_{50}	LC_{90}	χ^2
1	17.42 ± 4.61^{cd}	32.63 ± 4.04^{cd}	43.50 ± 4.13^{de}	51.11 ± 3.10^{c}	0.87	0.76	20.11 ± 0.034	0.000	871.14	2268.09	43.11*
2	10.81 ± 3.62^{abc}	17.30 ± 4.10^{ab}	24.97 ± 4.52^{b}	32.57 ± 5.12^{b}	0.87	0.76	10.37 ± 0.024	0.000	1509.42	3137.48	27.91
3	28.24 ± 1.96^{e}	35.84 ± 4.45^{d}	42.39 ± 2.28^{d}	47.83 ± 2.30^{c}	0.88	0.78	28.99 ± 0.020	0.000	1021.37	3426.26	12.92
4	7.60 ± 2.94^{ab}	13.04 ± 2.93^{a}	15.20 ± 2.33^{a}	19.70 ± 5.79^{a}	0.81	0.65	7.53 ± 0.015	0.000	2213.23	4273.60	21.80
5	4.32 ± 4.61^{a}	11.98 ± 6.14^{a}	17.42 ± 2.71^{a}	19.53 ± 2.65^{a}	0.72	0.52	6.21 ± 0.015	0.000	2131.16	3997.78	55.51*
6	15.26 ± 4.75^{bc}	23.85 ± 4.52^{bc}	32.57 ± 3.29^{c}	42.33 ± 3.66^{c}	0.91	0.83	14.89 ± 0.020	0.000	1167.88	2688.15	25.02
7	23.91 ± 2.94^{de}	40.17 ± 5.69^{d}	50.00 ± 4.34^{e}	60.93 ± 5.31^{d}	0.89	0.79	26.09 ± 0.038	0.000	636.06	1951.39	39.33*
8	30.46 ± 3.24^{e}	51.05 ± 4.18^{e}	59.82 ± 4.30^{f}	71.69 ± 2.90^{e}	0.89	0.79	34.14 ± 0.041	0.000	384.43	1576.55	41.13*
Azadirachtin	42.33 ± 3.66^{f}	54.26 ± 4.01^{e}	65.20 ± 2.98^{f}	76.02 ± 3.50^{e}	0.92	0.85	42.76 ± 0.036	0.000	206.42	1525.75	18.92
ANOVA	Df 8, 36 F 54.93	Df 8, 36 F 59.80	Df 8, 36 F 126.85	Df 8, 36 F 111.95							
Homogeneity	0.74	0.93	0.60	0.001							

Means followed by the same letter do not differ significantly using Tukey's test ($P \leq 0.05$) and complete regression equations; * χ^2 values are significant.

1, 3, and 6 showed ovicidal activity between 42 and 51% and were statistically similar (P value 0.065). The data of all the fractions showed homogeneity variance except at 1000 μg/mL concentration while using one-way ANOVA. The R^2 value exhibited concentration dependent activity. Minimum R^2 value was observed in fraction 5 which showed less than 20% ovicidal activity at maximum concentration. Higher concentration of the fraction increased the ovicidal activity. Maximum regression coefficient was observed in fraction 8 followed by fraction 7. Minimum regression coefficient value was noticed in fraction 5 (Table 2). All these data clearly indicated concentration-dependent activity. All the analysed regression data were significant (P value 0).

Minimum LC_{50} and LC_{90} values of 384.43 and 1576.55 μg/mL, respectively, were obtained in fraction 8 (Table 2). Fraction 4 showed maximum LC_{50} and LC_{90} values of 2213.23 and 4273.60 μg/mL, respectively. Fraction 5 had lower percent of ovicidal activity than the other fractions; in case of probit analysis, fraction 4 showed lower value than fraction 5. Fractions 1 and 7 showed less than 1000 μg/mL LC_{50} values. Fractions 1, 5, 7, and 8 showed significant χ^2 values.

4. Discussion

Hexane, chloroform, and ethyl acetate extracts of *C. guianensis* showed ovicidal activity against *S. litura*. This finding corroborates with the findings of Deepa and Remadevi [22] who reported that the petroleum ether, chloroform, ethyl acetate, methanol, ethyl alcohol, and acetone extracts of *Acacia concinna* and *Butea monosperma* showed ovicidal activity against lepidopteran insect, *Hyblaea puera*. Similarly, water extract exhibited ovicidal activity against *Sambucus ebulus* and *Tribolium confusum* [23]. Crude extracts with a mixture of compounds showed strong ovicidal activity against *S. litura* in this study. Similarly, many researchers around the world have reported many plant extracts with ovicidal activity. *Myrtus communis, Melaleuca alternifolia, Pimenta dioica, Syzygium aromaticum, Eucalyptus citriodora*, and *E. globulus* exhibited ovicidal activity against *Trialeurodes vaporariorum* [24]; *E. globulus* and *Syzygium aromaticum* showed ovicidal activity against *Tribolium castaneum* [25, 26]; and *E. camaldulensis* showed ovicidal activity against *T. confusum* and *Ephestia kuehniella* [27]. Methanol extract of *Celosia argentea, Ricinus communis, Mikania micrantha,*

and *Catharanthus roseus* reduced the egg hatchability in *Brontispa longissima* and maximum reduction was observed in *M. micrantha* [28]. Similarly, citronella oil reduced the egg hatchability up to 95% against *Helicoverpa armigera* [29].

Fractions from hexane extract showed ovicidal activity against *S. litura*. Fractions exhibited maximum ovicidal activity at lower concentrations than the crude hexane extract. This result corroborates with the findings of Jeyasankar et al. [30] who reported that ethyl acetate extract, its fractions, and isolated compound showed ovicidal activity against *S. litura*. Maximum activity was noticed at lower dose in the purified compound than the higher dose treated fractions and crudes extracts. In the present study, the presence of alkaloids, coumarin, and quinone in the hexane extract could be responsible for ovicidal activity against *S. litura*. Similarly, Maciel et al. [31] reported that the presence of different phytochemicals like tannins, triterpenes, and alkaloids in the ethanol extract of leaves and seeds of *M. azedarach* is responsible for ovicidal activity. In the present study, partially purified extract (fractions) showed maximum ovicidal activity against *S. litura*. Similar results were obtained by Alouani et al. [32] against mosquito larvae.

Hexane extract and fraction 8 exhibited ovicidal activity against *S. litura* with least LC_{50} values than the other extracts and fractions. In this study, hexane extract eluted fractions using hexane : ethyl acetate or ethyl acetate showed ovicidal activity. The present findings coincide with the findings of Baskar and Ignacimuthu [16] who reported that hexane extract fractions eluted with hexane : ethyl acetate from *C. guianensis* showed maximum ovicidal activity against *H. armigera*. Hexane extracts derived ethyl acetate fractions from *Atalantia monophylla* showed maximum ovicidal activity against *H. armigera* and *S. litura* [33, 34]. Similarly, fractions eluted using hexane : ethyl acetate from chloroform extract of *Clerodendrum phlomidis* showed maximum ovicidal activity against *Earias vittella* [14].

5. Conclusion

The present study clearly indicates that the hexane extract and its active fraction showed the least LC_{50} values against the eggs of *S. litura*. Further study is necessary to identify the active principle(s) responsible for the activity and to develop a new formulation to control the agricultural pests.

Conflict of Interests

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

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Review Article

Sex-Pheromone-Mediated Mating Disruption Technology for the Oriental Fruit Moth, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae): Overview and Prospects

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A great deal of progress has been made over the last three decades in research on pheromone-mediated mating disruption technology for the oriental fruit moth, *Grapholita molesta* (Busck). Pheromones can interrupt normal orientation, and the most likely mechanism of pheromone disruption, competitive-attraction (false-plume following), invokes competition between point sources of pheromone formulation and females for males. This technology, performed by broadcasting pheromones into orchards to disrupt mate finding, has been successfully implemented in oriental fruit moth control. Reservoir-style dispensers made of polyethylene tubes, which release pheromone throughout the full growing season, are the current industry standard. Although reasonably effective, they require labor-intensive hand application. Recently, a new formulation, paraffin wax, which maximizes competition between point sources of synthetic pheromone and feral females for males, was shown to have high disruption performance. As this formulation is highly effective, inexpensive, and easy to produce, further study and development are advisable. Increased understanding of the principles of mating disruption will aid in the design of more effective dispensers. Continued research is needed to meet grower concerns with regard to risk, efficacy, and cost and to identify other semiochemicals that can be applied to this delivery system. Greater knowledge of the integration of different biological control methods is therefore essential.

1. Introduction

The oriental fruit moth (OFM), *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae), is a key pest of stone and pome fruit in most fruit-growing areas of China, with the exception of Tibet [1–6]. Until recently, this pest has been primarily controlled by use of one or more broad-spectrum insecticides [7]. Issues associated with the widespread use of insecticides, including insecticide resistance, toxicity to natural enemies, worker safety, and food residue, have provided an impetus for research and development regarding alternative control technologies. The application of pheromone-mediated mating disruption technology has resulted in excellent control of this pest and could be an alternative to conventional insecticide use [2, 8]. This review presents the principle of pheromone-mediated mating disruption, summarizes the typical application of this technology, introduces its pheromone release device (pheromone dispenser), and discusses future directions for research and development.

2. Principles of Pheromone-Mediated Mating Disruption

Pheromone-mediated mating disruption controls insect species mainly by using chemicals involved in its own communication system [9]. Although it is not necessary to understand the mechanism underlying mating disruption to verify its efficacy, analysis of probable modes of action is useful to determine the reasons behind success and failure of various formulations [2].

Sex pheromones are specific chemicals released by females into the air to attract conspecific males for mating. Males follow the sex pheromone upwind to locate and mate

with the female [9]. However, if the air in an orchard is filled with synthetic sex pheromone, males would encounter high-dose artificial point sources of pheromone and low-dose calling females' point sources of pheromone during their upwind zigzag flight. As males preferentially orient toward artificial point sources of pheromone than pheromone plumes from calling females, they would be unable to accurately locate calling females and this would greatly modify moth flight tracks. Therefore, synthetic sex pheromone successfully prevents males from finding and mating with calling females. This sex-pheromone-mediated mating disruption technology can protect orchards from pests, including the OFM, and ultimately achieve long-term reduction of the pest population [2, 9]. Many studies have indicated that competitive attraction; that is, false plume following, may be a leading mechanism underlying disruption of OFM mating by synthetic sex pheromones [10–13] and the most feasible principle of pheromone disruption [2, 14–16] including sensory adaptation or central nervous system habituation on males, while sensory imbalance affects the female plume and the way in which it is perceived by males. When encountering a high concentration of formulated pheromone above that produced by calling females, males may show an increase in response or complete abolition of responsiveness to subsequent pheromone released by females, because of adaptation of peripheral receptors on the antennae or habituation at the central processing level. In lepidopterous insects, pheromones are generally comprised of more than a single component with a very narrow range of ratios. An imbalance in sensory input may be produced and male response to the natural ratio of the components may be decreased by the disproportionality of blends or lack of partial components. In addition, reduction of female copulation propensity may be a secondary mechanism of mating disruption that affects the mating behavior of females in addition to that of males [17]. As an indicator of high competition, the high levels of sex pheromone perceived by females are unsuitable for reproductive success, thus restricting their receptiveness to copulation and causing the loss of female receptivity to mating. Mating of females may be adversely affected following sex pheromone autoexposure due to abnormal behavioral activity, which is thought to be because the preexposed females may be unable to sense the aphrodisiac pheromone of conspecific males and/or antennal sensitivity, which is interpreted as the requirement for male aphrodisiac, which plays a role in courtship, for the occurrence of adaptation of antennal responses, following sex pheromone preexposure.

Attention should be paid to the interplay between these principles. With regard to male response threshold raised by encountering a high-dose, artificial source of pheromone, subsequent male responses minimize the odds of detecting females that emit relatively low levels of pheromone, and in turn the enhanced competition effect of the two point sources of pheromone may encourage males to visit a high-dose point-source formulation than to locate and mate with a female. Taken together, these principles are helpful to optimize the effect of mating disruption technology by guiding the behavioral peculiarities of the insect to be managed, its spatial distribution, the type of formulation employed, and the rate of formulation application [16, 18].

3. Application of Sex-Pheromone-Mediated Mating Disruption

The sex pheromone of OFM was determined to be a mixture of four components [19]: (*Z*)-8-dodecenyl acetate (*Z*8-12:Ac), (*E*)-8-dodecenyl acetate (*E*8-12:Ac), (*Z*)-8-dodecen-1-ol (*Z*8-12:OH), and dodecanol (12:OH). As a disruptant, only the (*Z*)- and (*E*)-isomers of the acetate were used in early work performed in Australia [20] and USA [21, 22], indicating that OFM was highly susceptible to communication disruption. Later studies [19, 23] indicated that the addition of *Z*8-12:OH and 12:OH reduced the amount of pheromone required for disruption and for accurate location of hosts over short distances, respectively. Field results in a number of countries [2, 8, 9, 20, 24–34] indicated that this technology was capable of truly managing pesticide-resistant populations throughout the whole growing season and was therefore fully equivalent or superior to conventional pesticides. Therefore, sex-pheromone-mediated mating disruption technology could provide complete crop protection [2, 35].

4. Pheromone Dispenser

Based on the above discussion, use of a good pheromone dispenser plays a key role in achieving high-performance mating disruption. First, an ideal pheromone dispenser should remain effective for a prolonged period, not waste active ingredients, be inexpensive to produce, be easy to use in the field, and be nontoxic [36]. Furthermore, pheromone dispensers should be amenable to use at varying densities and deployment dates according to pest pressure. In addition, it should achieve the availability of a controlled release device to encourage growers to adopt mating disruption technology [37–41].

4.1. Common Pheromone Dispensers. If competitive attraction is the foremost mechanism underlying mating disruption, as suggested by recent studies [10–12], various pheromone dispensers would be desirable as shown in Table 1. The three most popular types are illustrated here, that is, hollow fiber dispensers, polyethylene tube dispensers, and sprayable formulations of microscopic capsules.

In the 1970s, Cardé et al. (1977) deployed 1700 hand-applied hollow fiber pheromone dispensers per ha according to this behavioral modification tactic and achieved successful control of OFM, thus indicating that this is a promising alternative to broad-spectrum neurotoxins [21, 42].

In the 1990s, hand-applied polyethylene tube dispensers, such as Isomate-M, M 100, and M Rosso (Pacific Biocontrol Co., Litchfield Park, AZ) became available for commercial use for disruption of OFM [25, 27, 43–47]. These dispensers were filled with 75–250 mg of OFM pheromone and applied by hand at 500–1000 units/ha (corresponding to 1–4 dispensers/tree), and pheromone release per dispenser varied between ca. 600–1000-fold as much as that produced by a calling female. Accumulating evidence showed that

Table 1: Use of the pheromone-mediated mating disruption technique for oriental fruit moth in various countries [2, 8, 9, 20, 24–34].

Country	Type of dispensers	Density (/ha)	Efficacy and extension
France	Polyethylene tube	1000	The two kinds of dispensers were the most effective and simple to use.
	Polyethylene bulb	500	
US	Polyethylene tube	1000	The pheromone-treated peach and nectarine orchards in California and Virginia extended from 600 ha in 1987 to 4000 ha in 1990.
	Microcapsule	—	Male OFM captures were reduced by 77–98% in 35 d, and the formulated pheromone significantly disrupted male orientation.
Japan	—	—	The effect of mating disruption was greater than that of trapping.
Korea	—	—	The effectiveness of the disruption technique was enhanced through sequential suppression year after year at the same site.
Australia	400 mL microcentrifuge tube	—	In this extensive 3-year trial, use of pheromone treatment increased from 25 to 40 ha and confirmed that conventional insecticides could be replaced by mating disruption from the viewpoint of cost and efficacy. These successful tests encouraged use of mating disruption in over tens of thousands of hectares in the peach-growing districts in New South Wales and Victoria.
	Polyethylene capillary	500	Male OFM capture was reduced by 77–98%.
		1000	OFM males preferred untreated orchards adjacent to pheromone-treated orchards.
	Polyethylene tube	—	Pheromone treatment reduced the capture of moths in pheromone-baited traps by an average of 98%, suggesting a high level of disruption.
	—	—	Application of mating disruption barriers on pears during two consecutive seasons provided sufficient control of OFM on peaches, and this mating disruption barrier treatment was able to reduce the number of OFM caught in all experimental peach blocks. Extending the mating disruption treatment area for 54–60 m into the neighboring pear block significantly reduced the edge damage in mating disruption-treated peaches in the first season and almost eliminated OFM damage in the second season.
	—	—	Mating disruption was a viable alternative to conventional insecticides.
China	Rubber septa	—	In Liaoning province, Eastern China, the percentage of infested fruits in the pheromone-treated orchards was reduced to 50% and 72% compared with the insecticides-treated orchards in 1981 and 1982, respectively; this technology was extended on a large scale.
		1050	In pear orchards of Shanxi province, Northern China, male OFM orientation was disrupted by 97.43% and the percentage of infested fruits was reduced by 74.72% in 2009.
		750	In pear orchards of Shanxi province, Northern China, male orientation was disrupted by 81.83% and the percentage of infested fruits was reduced by 56.43% in 2009.
		3000	In peach orchards of Shanxi province, Western China, after 37 d, under no insecticide, male orientation was disrupted by 93.46% and the percentage of infested fruits was reduced by 73.72% in 2007.
	Twist tie	374	In peach orchards of Shanxi province, Western China, after 37 d, under no insecticide, male orientation was disrupted by 97.19% and the percentage of infested fruits was reduced by 81.61% in 2007.

the relatively high densities of pheromone release sites at common overall release rates of pheromone per ha could achieve superior disruption for most moths [12, 13, 23, 48–53]. However, because of the appreciable costs of purchase and labor for hand application, polyethylene tube dispensers have not been widely adopted in some production systems and in many developing countries [53].

Early in the 21st century, sprayable formulations of microscopic capsules that release pheromone for prolonged periods were developed and shown to be effective against OFM when properly applied [4, 46, 54]. They were generally applied with standard air-blast sprayers. Not only they were considered a cost-saving alternative to hand-applied dispensers, but also they can be tank-mixed and coapplied with other orchard management chemicals [4, 54]. However, they maintained effectiveness for only 2–4 weeks and required more frequent applications than hand-applied reservoir dispensers [4, 47, 54]. Other drawbacks also included wash-off of microcapsules by heavy rain and degradation of active ingredients by UV irradiation [55, 56].

In addition, due to their ease of production and constant release rate, rubber septa dispensers are common and effective means of controlling OFM in China. Special lures for mating disruption are directly suspended in the upper third of the tree canopy at 200–400 units/ha without traps. However, the cost associated with this method is ca. US$180/ha per year, so they are unsuitable for common orchards in China. In addition, the rubber septa age rapidly, the duration of pheromone release is short, and hand application is expensive [57, 58]. Although they are mainly used for baiting monitoring traps, many research groups spent considerable time and effort to develop more reliable and efficient methods of using rubber septa [32–34, 57, 58].

4.2. New-Style Pheromone Dispenser: Paraffin Wax. Dispensers in which insect sex pheromones are mixed at the required concentrations into paraffin wax emulsions have been used in USA for almost 16 years [59, 60]. Two typical wax-paraffin dispensers, that is, Confuse-OFM and SPLAT-OFM, are described below.

Confuse-OFM resembles white, liquid glue and is applied using squirting devices, such as forestry paint marking guns and plastic squirt bottles [53, 61]. Use of this type of dispenser was shown to inhibit capture of male moths in pheromone traps and shoot damage as effective as Hercon (Hercon Environmental, Emigsville, PA) and Consep (Consep, Inc., now Suterra LLC, Bend, OR) hand-applied pheromone dispensers [59]. The University of California at Davis patented this emulsion (U.S. Patent 6,001,346) [62] and it was later commercially developed by Gowan Co. (Yuma, AZ) [53].

SPLAT-OFM consists of microcrystalline wax emulsified in water and so it can be pumped from a storage reservoir and sprayed onto the crop, and it shows long-lasting adhesion of dispensed particles on plant surfaces [63]. Stelinski et al. (2006) reported that mechanical application of SPLAT-OFM could save time and labor for mating disruption of OFM in apple orchards [64]. In 2003, ISCA Technologies, Inc. (Riverside, CA), patented this wax emulsion, and extensive testing of this technology was later performed along with adaptation for a variety of pests and crop systems (ISCA Technologies, Riverside, CA). ISCA Technology's Specialized Pheromone & Lure Application Technology (SPLAT) has been granted a federal registration for OFM control by the U.S. Environmental Protection Agency, and it is now commercially available as SPLAT OFM 30 M-1 [53].

4.2.1. Efficacy. Optimization of mating disruption requires that the density and size of droplets, pheromone release rate, and duration are appropriate for the biology of the targeted pest [13, 14]. Researchers can not only easily manipulate the size, density, and distribution of wax droplets, but also flexibly investigate how moths could be actually disrupted [65].

In 2005, male OFM orientation was shown to be disrupted more effectively by deploying ~8,000 0.1-mL drops of SPLAT-OFM per ha (each containing ca. 1% of the total pheromone active ingredient of a standard Isomate dispenser) compared with the label rate of 500 Isomate-M Rosso dispensers per ha, probably because sufficient point sources of pheromone were provided for optimal disruption of OFM with the typical deployment density of pheromone twist ties [51]. In 2006, male OFM orientation was shown to be disrupted by 98% relative to untreated control plots during the whole season using SPLAT-OFM, and either increasing the size of wax drops above the average volume of 0.04 mL achieved by the initial applicator prototype or reformulating the wax to allow for a higher initial pheromone loading concentration for longer release over time, especially in hot temperatures, could maintain efficacy and improve longevity [64]. In 2007, two applications of Confuse-OFM were shown to be as effective against OFM as one application of Isomate-M 100. A new emulsified wax formulation, Wax Dollops, was developed in 2007 with a release rate exceeding a 5 mg/ha/h threshold and duration of action that is twice as long as Confuse-OFM. One application of 3 mL dollops (ca. 590 dollops per ha) provided season-long (ca. 15 weeks) control, which was equivalent to the effects of Isomate-M 100 and Confuse-OFM applied as described above [53].

4.2.2. Advantages. Paraffin wax dispensers are inexpensive and easy to produce. Paraffin wax consists mostly of water and wax, which is a byproduct of petroleum refining, and it is therefore readily available and inexpensive [63, 64]. Wax emulsions can easily be increased proportionally and manufactured on a large scale with minimal labor [53]. Therefore, commercial production of wax emulsions should be cheaper than other currently available hand-applied formulations.

Paraffin wax is a viscous homogenate that hardens on crop foliage or branches once applied and therefore can act as a long-lasting discrete source of pheromone emission. Delwiche et al. (1998) reported that one of the initial formulations (30% paraffin wax emulsified in water, vitamin E, soy oil, and antioxidant) was as effective as Shin-Etsu, Isomate-M 100 polyethylene-tube dispensers for 75 days in the field [61]. Subsequently, one application of a more viscous version of the above-described paraffin wax dispenser provided the same level of season-long disruption of OFM as Isomate-M 100 dispensers and could be hand-applied once in less time than Isomate-M 100 dispensers [50].

Paraffin wax dispensers are rapidly applied mechanically, and there is a cost-saving advantage to mechanical application [51–53, 60, 65]. For example, SPLAT can be easily applied with a machine forming numerous discrete point sources per area of crop [64]. A single operator can treat a hectare of crop with the current mechanized applicator in ca. 20 min, which is approximately 3.4-fold faster than hand-application of Isomate-M Rosso dispensers by three people [50]. Therefore, SPLAT-OFM currently represents an economical alternative to hand-applied reservoir dispensers for high-performance mating disruption of OFM.

In addition, the flowable, adhesive, and dispersible emulsified wax can be applied with a wide range of deposit sizes and spatial distributions [53]. Furthermore, paraffin wax dispensers contribute to effective disruption of communication for other moth species [13, 23, 48–52] and are not phytotoxic, so they do not damage foliage and/or mark fruit [66]. In addition, insecticides have been incorporated into emulsified wax to produce effective attracticide formulations (ISCA Technologies).

4.2.3. Drawbacks. Pheromones are costly—for season-long control of OFM, the cost of Confuse-OFM was three times that of Isomate-M 100 (148 g AI/ha vs 57 g AI/ha) [53], and 160 g/ha of pheromone of SPLAT-OFM exceeded the 125 g AI/ha label rate of the Isomate-M Rosso reservoir dispenser [63].

In contrast, hand-application of dispensers is both time-consuming and expensive. Season-long control of OFM requires two applications of Confuse-OFM, and its application is laborious because this liquid formulation requires care and time for application to the tree bark [53]. In addition, even though SPLAT-OFM is applied mechanically, machine applicators are not affordable for individual growers, so the initial investment in the applicator for application of SPLAT should be provided by the manufacturer and/or distributor [64].

5. Future Prospects

As a major fruit pest [1], OFM is a long-standing target for the development of mating disruption programs [19, 21, 67, 68]. Therefore, accumulating evidence of the reliable and economic applications of pheromone-mediated mating disruption will lead to more widespread adoption of this technology.

First, it is necessary to determine the actual costs of OFM pheromone dispensers as well as the relations between the total costs for one or two applications of pheromone dispensers (materials and labor) in comparison with three insecticide and/or miticide applications (materials and labor) [2].

Second, laboratory assays are required to predict the effects of various types of dispensers on OFM behavior in the field, because the development of mating disruption technology still relies on repeated field trials and therefore remains both costly and slow [69].

Finally, sex-pheromone-mediated mating disruption is currently specific for male OFM, and we should develop other semiochemical-based methods, such as plant volatiles [70, 71], directly targeting females as the most important complement to mating disruption. In addition, more emphasis should be placed on the integration of different biological control methods, such as use of microbial pesticides [72], to reinforce the effects of behavior-modifying chemicals. All biological methods, such as black light [73], are to some extent species-specific and do not cover all pests associated with a crop, so it will be necessary to develop new mating disruption technology for OFM and other lepidopterous pests in orchards [17, 69, 74].

Conflict of Interests

The authors declare that there is no conflict of interests.

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Retraction

Retracted: Climatic, Regional Land-Use Intensity, Landscape, and Local Variables Predicting Best the Occurrence and Distribution of Bee Community Diversity in Various Farmland Habitats in Uganda

Psyche

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The article titled "Climatic, Regional Land-Use Intensity, Landscape, and Local Variables Predicting Best the Occurrence and Distribution of Bee Community Diversity in Various Farmland Habitats in Uganda" [1], published in Psyche, has been retracted as it was found to include erroneous data. Its findings and conclusion cannot be relied on.

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[1] T. Munyuli, "Climatic, regional land-use intensity, landscape, and local variables predicting best the occurrence and distribution of bee community diversity in various farmland habitats in Uganda," *Psyche*, vol. 2013, Article ID 564528, 38 pages, 2013.

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Research Article

The Effect of Conspecific Density on Emergence of *Lestes bipupillatus* Calvert, 1909 (Odonata: Lestidae)

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Conspecific density may influence adult recruitment and consequently population dynamics. Several studies have shown the density dependence of larvae growth rates in Odonata. However, few studies studied how conspecific density influence final instar larvae emergence date decisions. Considering that larvae may choose the date of emergence, the present study investigated if density affects larvae choice. For this, we reared eight final instar larvae in individual aquaria and other 24 larvae in aquaria with three larvae each. This way, we simulated environments with low and high larval densities. We then noted the days that larvae took to emerge and compared it between low and high density groups. The results showed that larvae seem to emerge earlier when in high densities (Mann-Whitney, $U = 10.000$, $P = 0.03$). These results support the hypothesis that damselfly last instar larvae may postpone or hasten emergence in response to the social environment and related constraints.

1. Introduction

Natural environments may exhibit large temporal fluctuations, which entail a major challenge for animal species. Temporary pools comprise a harsh environment, inhabited by a unique fauna with physiological and behavioral adaptations that enable development and survival [1]. Reductions in water levels in temporary pools may affect species population dynamics, since density should increase.

Population dynamics are influenced by life history features such as individual development, survival, fecundity, and dispersal rates amidst environmental fluctuations. Variation in such features may be associated with density-dependent processes [2–6]. In insects, adult population dynamics are usually affected by larval density that may decrease or increase adult emergence rates [7, 8].

In Odonata, increasing density among conspecifics may shorten life cycle [9], influence larval growth rates, and affect species voltinism [1]. The density dependence of larvae growth rates in odonates is well studied [1], but there is no evidence of how conspecific density may determine the emergence rate of final instar larvae. The increased density during the reduction of water level could be an indicator cue of the drying out process. This mechanism could enable some species of Odonata to colonize and complete their life cycles in temporary pools.

Since final instar larvae of Odonata may postpone emergence, the date of emergence can determine individual body size, fecundity, and reproductive success [1] and may be critical to complete the cycle in temporary ponds. Thus, we tested if conspecific final instar larval density influences the date of emergence in the tropical species *Lestes bipupillatus*

Calvert, 1909 (Zygoptera: Lestidae). Lestids are good models for this kind of study since they inhabit temporary pools and must carry adaptations to such environment [1, 10–12].

2. Material and Methods

We collected last instar larvae in a temporary pond near the Ecological Reserve Horto Florestal in Assis, SP, Brazil (S 22°37′46.9″/W 50°24′11.7″) on 19 May 2007. This reserve is a conservation area with a mixture of native Atlantic Forest vegetation and Neotropical Savanna vegetation.

To test whether the density affects the time of emergence, we simulated two situations in laboratory: (i) low density, with one individual per aquarium; and (ii) high density, with three individuals per aquarium. We considered eight replicas for each situation. Each aquarium had 500 mL of capacity and was filled with 300 mL of filtered water collected in the habitat of larvae. The aquaria were wrapped with white paper to prevent visual contact between larvae and were provided with wood sticks for individuals to climb during the rearing process.

During the experiment, the aquaria were maintained in a cool room with 12 : 12 photoperiod. The aquaria were placed inside a vial filled with water to guarantee temperature constancy among replicas. The positioning of each aquarium in the vial was randomly sorted.

We checked for emergence each 12 hours and we finally compared the number of days that the two groups of larvae took to emerge since the collection date. For the high density group, we sorted eight individuals to represent the group. Differences between the median of emergence time of individuals at high and low densities were assessed using the Mann-Whitney U test.

3. Results and Discussion

The results show that high conspecific density decreased the number of days until emergence (Mann-Whitney, $U = 10.000$, $P = 0.03$, Figure 1). As the larvae at high densities emerged earlier, we can assume that, when there is low conspecific density, the larvae may delay emergence. These results show how density may influence adult recruitment and the number of flying reproductive individuals in a given time.

Based on this information, we can consider the fact that high density may force larvae to hasten emergence and impose a great impact on population dynamics, since larvae that emerge earlier are usually smaller and have a lower reproductive success [1, 12]. We can also consider extrinsic features related to species ecology and the peculiar habitat which they inhabit. Since this study collected *L. bipupillatus* larvae on a temporary pond, another possible selective force could be pond dryout [12], which may result in larvae aggregation with the decline of water level. In this case, the high density is an indicator that water level is dropping and the early emergence occurs to avoid death due to the low volume of water, high temperatures, and low dissolved oxygen.

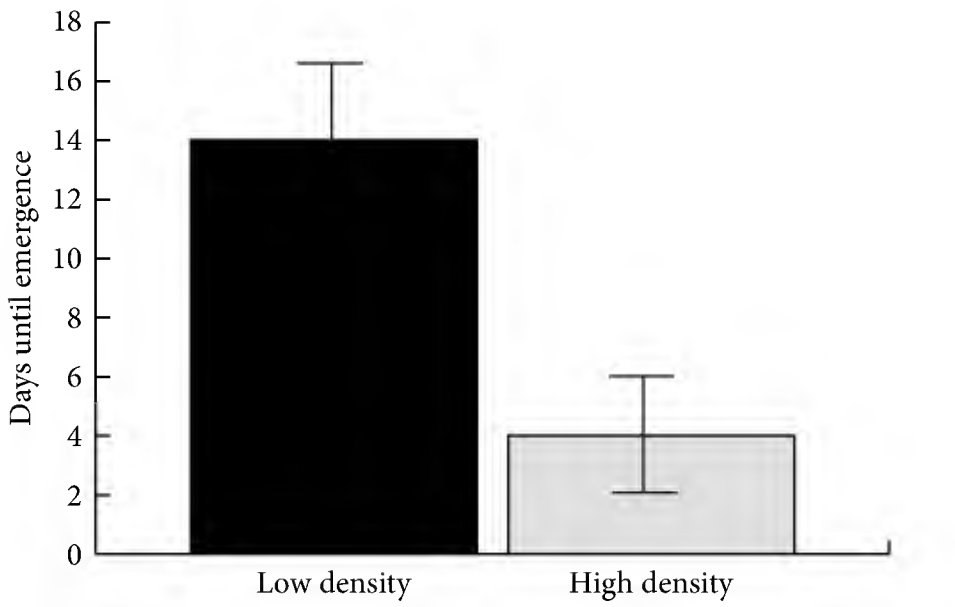

Figure 1: Days until emergence of last instar larvae reared with low and high conspecific densities.

The earlier emergence when in high densities may also be an evolutionary response to conspecific interactions as cannibalism, since odonate larvae usually feed on conspecifics [13–16] or competition, since they can be aggressive towards conspecifics and even harm or kill neighboring larvae [1, 16].

4. Conclusions

In conclusion, the experiment allows us to suggest that damselfly last instar larvae may postpone or hasten emergence in response to the constraints related to the social environment and water conditions. Although other studies show that many variables may affect development, and consequently emergence [1, 17], here we show that *L. bipupillatus* last instar larvae make decisions regarding emergence time, independently of previous development. This can give base for future perspectives, regarding other environmental variables and the intrinsic effects on adult survival and reproduction. We suggest that studies should now focus on the outcomes and handicaps of final instar larvae emergence syndromes in a set of species.

Conflict of Interests

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

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Research Article

Diversity and Composition of Beetles (Order: Coleoptera) of Durgapur, West Bengal, India

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A survey of beetle faunal diversity and composition was studied in Durgapur Municipal Corporation, Durgapur, West Bengal, from January to December 2012. Beetles were collected using standard trapping methods from three different sites selected on the basis of their specific habitat differences, identified up to the level of family, and counted monthly. A total of 9 families were reported from the study site. The second site, that is, Site B, showed the highest diversity. It is also noted that the highest diversity was found during monsoon in all the three sites.

1. Introduction

Coleoptera is an order of insects commonly called beetles. The word "coleoptera" is from the Greek *keleos*, meaning "sheath," and *pteron*, meaning "wing," thus "sheathed wing." The reason for the name is that most beetles have two pairs of wings, the front pair, the "elytra," being hardened and thickened into a sheath-like or shell-like protection for the rear pair and for the rear part of the beetle's body. The order Coleoptera includes more species than any other order, constituting almost 25% of all known life-forms [1–3]. About 40% of all described insect species are beetles (about 400,000 species) [4] and new species are discovered frequently. Some estimates put the total number of species, described and undescribed, at as high as 100 million, but a figure of 1 million is more widely accepted [5]. The diversity of beetles is very wide. They are found in all major habitats, except marine and the Polar regions. There are particular species that are adapted to practically every kind of diet. The family Scarabaeidae is the largest family of insects which contains more than 30000 species in the world [6]. Coleoptera are found in nearly all natural habitats, that is, vegetative foliage, from trees and their bark to flowers, leaves, and underground near roots, even inside plants like galls, tissue, including dead or decaying ones [7]. About 3/4 of beetle species are phytophagous in both the larval and adult stages, living in or on plants, wood, fungi, and a variety of stored products, including cereals, tobacco, and dried fruits. Because many of these plants are important for agriculture, forestry, and the household, the beetle can be considered a pest [8]. Beetles are not only pests but can also be beneficial, usually by controlling the populations of pests. One of the best, and widely known, examples is the ladybug or ladybird (family Coccinellidae). Both the larvae and adults are found feeding on aphid colonies. Other ladybugs feed on scale insects and mealybugs. If normal food sources are scarce, they may feed on other things, such as small caterpillars, young plant bugs, honeydew, and nectar [9]. Ground beetles (family Carabidae) are common predators of many different insects and other arthropods, including fly eggs, caterpillars, wireworms, and others [10]. Dung beetles (Coleoptera, Scarabaeidae) have been successfully used to reduce the populations of pestilent flies and parasitic worms that breed in cattle dung [9]. Dung beetles are taxonomically as well as functionally very important component of terrestrial ecosystem [11].

This study focuses on the diversity of beetles in Durgapur Municipal Corporation. The area is divided into three study sites in order to get an idea on the variety of beetles found.

The study is restricted to the family level of the order Coleoptera. 9 distinct families of beetles were reported from the three sites over a long one-year survey.

Usually diversity studies are conducted in ecologically sound areas with special focus on insects as they are the most diverse group among fauna. Durgapur is an industrial area of major importance in West Bengal. It has large power plants along with cement and iron factories. It has alarming pollution status. Thus it is important to survey the floral and faunal assemblage in this area. A study of the most diverse group of insects, that is, beetles, not only will help to assess the diversity of this area but also will help to carry out further studies to conserve the biodiversity of this industrial belt.

Table 1: Presence and absence of beetle families in three sites.

	Site A	Site B	Site C
Scarabaeidae	+	+	−
Carabidae	+	+	+
Chrysomelidae	+	+	−
Coccinellidae	+	+	−
Borydae	−	+	−
Lycidae	−	+	−
Curculionidae	−	+	−
Hydrophilidae	−	−	+
Derodontidae	−	−	+

2. Materials and Method

2.1. Study Area. A study was conducted from January to December 2012 at three different sites of Durgapur Municipal Corporation, Durgapur City, West Bengal, India. The geographical location is 23.30 N and 87.20 E with an altitude of 68.9 meters. Durgapur is about 220 Km from Calcutta, capital city of West Bengal. Though Durgapur is an industrial belt, the industrial sector is strictly demarcated from the main city area, which supports good floral assemblage. The metropolitan area is divided into three basic sites for the present study.

Site A is the college campus and its surrounding area that is dominated by Shal tree (*Shorea robusta*). Thus the floral composition is specific and constant.

Site B is the township area that mainly has residential complexes with gardens that support diverse floral composition that usually changes with season.

Site C is a wetland located near Amravati, surrounded by grasslands supporting different vegetation.

2.2. Field Method. Beetle sampling was done fortnightly from the three sites. For good collection two distinct standard methods were used. Pitfall traps were set up in all the three sites and were monitored every day. Light traps were also used specially in Site B. Apart from these, handpicking is also done. Sometimes shrubs and tree branches were heavily shaken so that beetles may fall on already spread large white sheets. After collection each specimen was preserved in 4% formalin and stored in small vials with proper labeling. Identification up to the family level was done using standard identification manual [12, 13].

2.3. Data Analysis. As beetles are reported to be the diverse group of insects, main focus of the present study is to estimate the diversity of beetles in this region. Different diversity indices were calculated, of which the widely used Shannon diversity indices were the most important, as it is widely accepted that all species at a site, within and across systematic groups, equally contribute to its biodiversity [14, 15]. A comparison of the diversities in the three different sites was also evaluated. These estimates were calculated using the standard software PAST. Graphical representation of monthly variation of beetle diversity was done using MS Excel.

3. Results and Discussion

After a long one-year study, 9 distinct families were identified from the three study sites (Table 1). These are Scarabaeidae, Carabidae, Chrysomelidae, Coccinellidae, Borydae, Lycidae, Curculionidae, Hydrophilidae, and Derodontidae. The last two families were strictly restricted to Site C, that is, the wetland. Families Borydae, Lycidae, and Curculionidae are reported only from Site B.

The total numbers of beetle of each family for each site are given in Figures 1, 2, and 3.

Diversity analysis study reveals that in Site A the Shannon Diversity indices gradually increase from January (1.18) and reaches the peak by June-July (1.35) and then slowly decrease to the end of the year (Table 2). Lowest value of Shannon Diversity is noted in the month of November (1.10). Similarly the Simpson (D) index is highest in the months of June-July (0.73) and lowest in November (0.59). Evenness values are also in accordance with the other diversity indices.

Similar studies in Site B reveal that Shannon Diversity is highest in the month of July (1.85) and lowest in the months of October-November (1.75). The dominance and evenness values also indicate a similar trend, that is, higher values in July and lower values in the months of October-November (Table 3).

Diversity analysis of Site C predicts that the Shannon Diversity index is more or less within a range of 1.05–1.10 from January to August but is low in the winter months. The other indices are also similar for this site (Table 4).

When the diversity indices were compared for all three sites together (Table 5), it predicted that Site B has the highest diversity, that is, Shannon Diversity (1.81), is and low for site C (0.66); the Dominance_D is low in Site B and high in Site C.

The shared species statistics between the three sites is done by Bray-Curtis cluster analysis and the Bray-Curtis similarity index is also calculated (Table 6). The Bray-Curtis similarity index shows 69.3% similarity between Site A and Site B and 12% similarity between Site A and Site C, whereas minimal similarity is seen between Site B and Site C (7%).

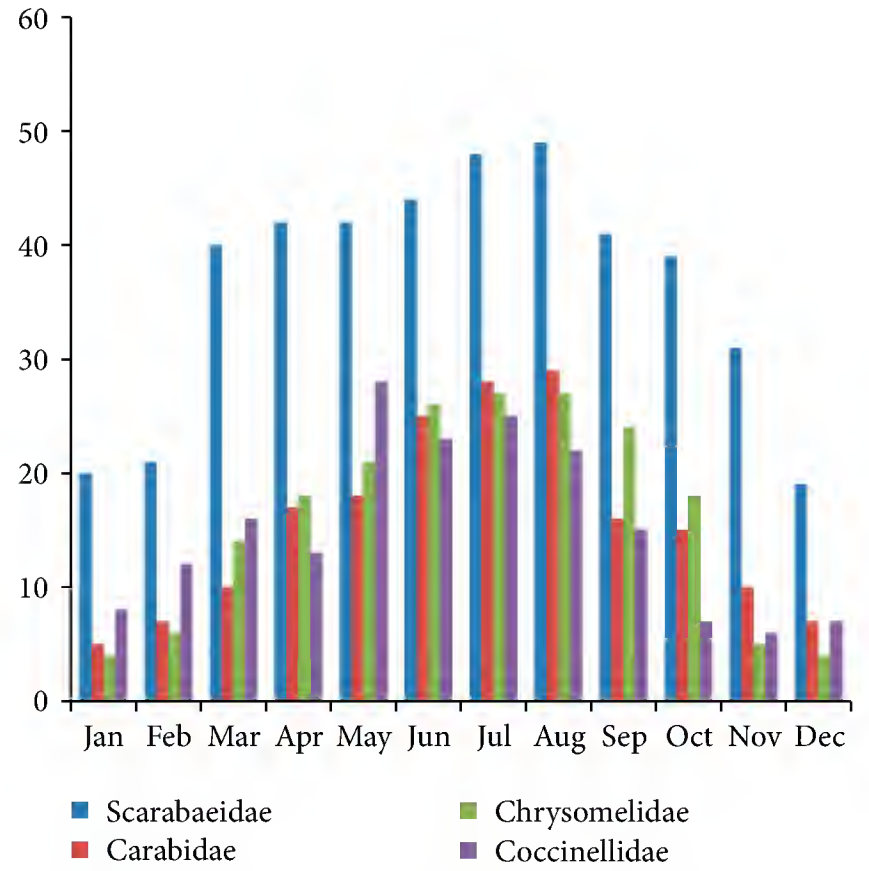

Figure 1: Total number of beetles of each family throughout the year at Site A (College Campus and surrounding).

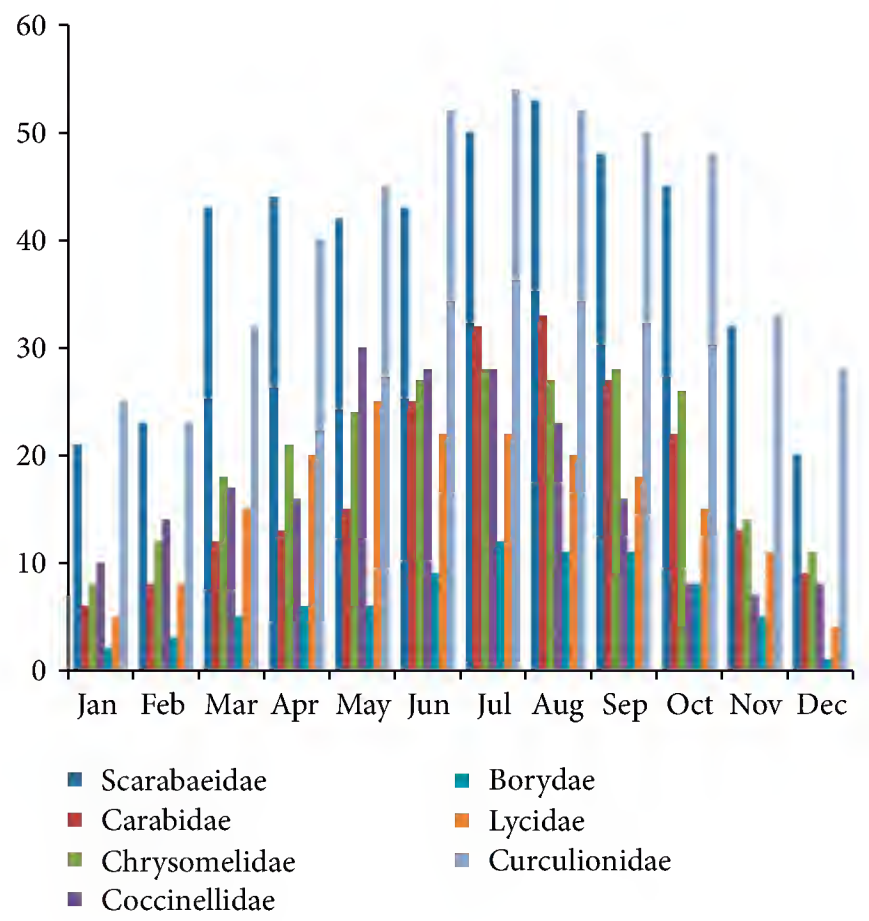

Figure 2: Total number of beetles of each family throughout the year at Site B (residential area).

Table 2: Diversity indices of Site A.

Site A	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Families	4	4	4	4	4	4	4	4	4	4	4	4
Individuals	37	46	80	90	109	118	128	127	86	79	52	37
Dominance_D	0.37	0.32	0.34	0.31	0.28	0.27	0.27	0.28	0.32	0.34	0.41	0.35
Simpson_1-D	0.63	0.68	0.66	0.69	0.72	0.73	0.73	0.72	0.68	0.66	0.59	0.65
Shannon_H	1.18	1.26	1.23	1.27	1.33	1.35	1.35	1.34	1.27	1.22	1.10	1.21
Evenness_e^H/S	0.81	0.88	0.86	0.89	0.95	0.96	0.96	0.95	0.89	0.84	0.75	0.84

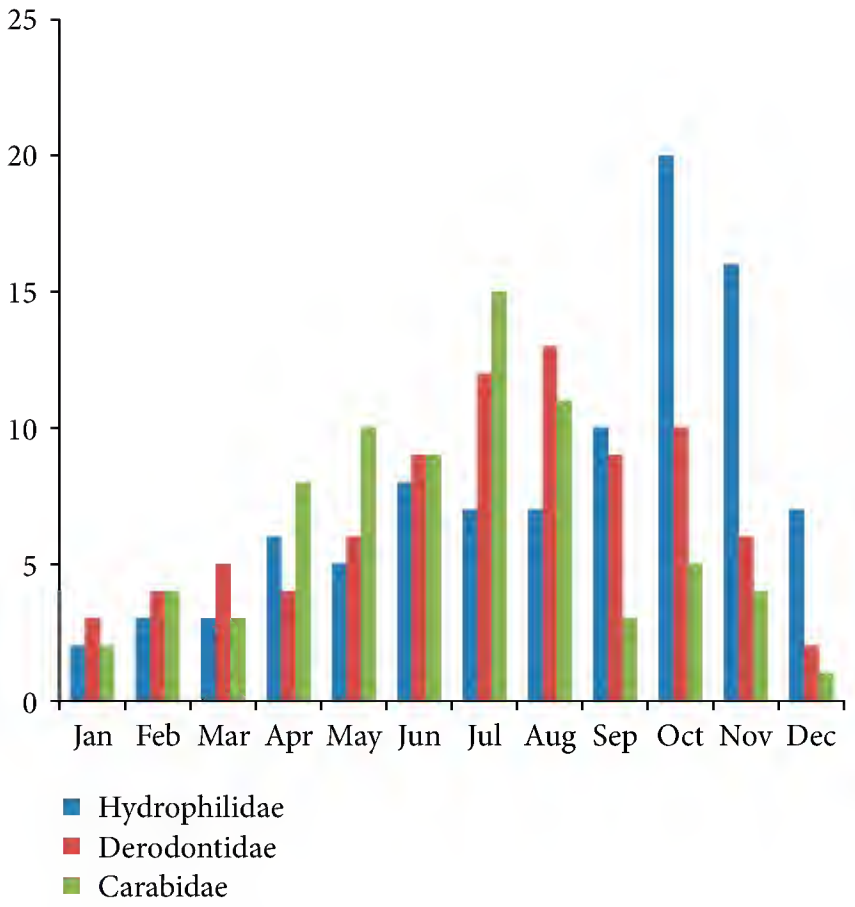

Figure 3: Total number of beetle of each family throughout the year at Site C (wetland).

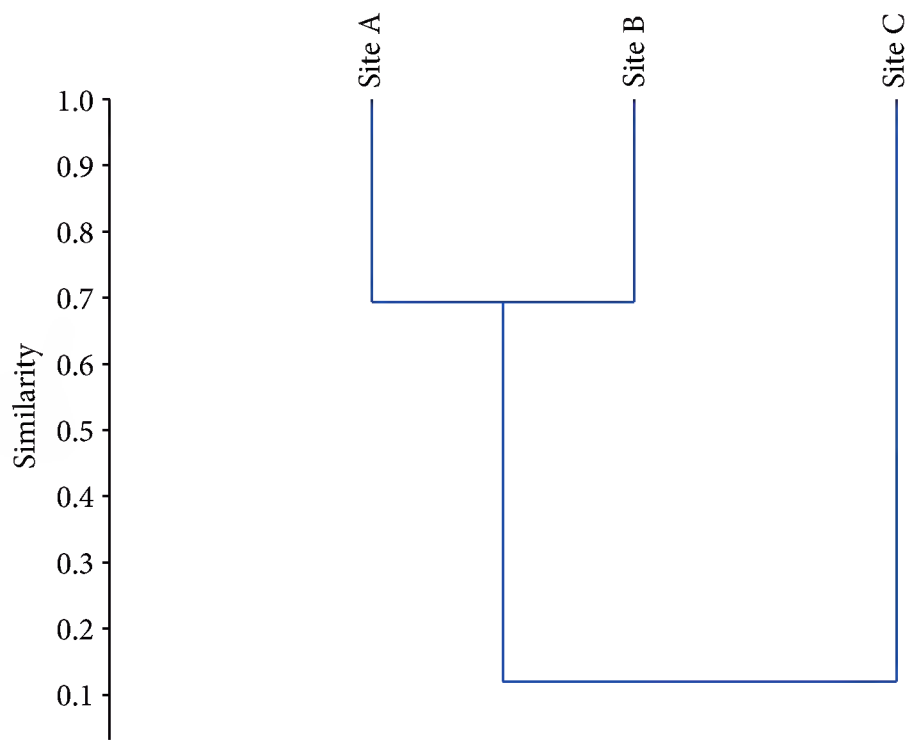

Figure 4: Bray-Curtis cluster analysis (single linkage).

The Bray-Curtis cluster analysis data shows that Site A and Site B form a small cluster and Site C is joined to it through a bigger cluster (Figure 4). This also suggests that Site A and Site B are similar in faunal composition rather than Site C.

4. Conclusions

It can be concluded that Durgapur though being an industrial city harbors a diverse variety of beetles. The most obvious reason is that being an industrial city, Durgapur has rich floral diversity that can support large growth of fauna. Even the municipality is well aware of the threats of an industry. As a result, the industrial belt is totally cut off from the main city centre.

The present study demonstrates that Site A and Site B are much diverse than Site C. Each of the sites shows highest diversity (as obtained from the calculated diversity indices) in June-July (monsoon) compared to Site C. Thus monsoon is the time when maximum beetles are found. It is known that most animals prefer monsoon as their breeding season as it is favorable and resourceful for their proper growth and survival. This study predicts that beetles are no exception to this occurrence. It is also true that insects usually avoid harsh winter through diapause, thus diversity of beetles in all three sites are least in winter months.

The floral composition of Site C is distinct from the other two sites. Only three families, namely, Hydrophilidae, Derodontidae, and Carabidae, are reported. The diversity indices also demonstrate a low diversity profile with high

Table 3: Diversity indices of Site B.

Site B	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Families	7	7	7	7	7	7	7	7	7	7	7	7
Individuals	77	91	142	160	187	206	225	220	198	172	115	81
Dominance_D	0.22	0.19	0.19	0.19	0.18	0.17	0.17	0.17	0.18	0.20	0.20	0.22
Simpson_1-D	0.78	0.81	0.81	0.81	0.82	0.83	0.83	0.83	0.82	0.80	0.80	0.78
Shannon_H	1.69	1.79	1.78	1.79	1.82	1.84	1.85	1.84	1.82	1.75	1.75	1.66
Evenness_e^H/S	0.78	0.86	0.85	0.85	0.88	0.90	0.91	0.90	0.88	0.83	0.82	0.75

Table 4: Diversity indices of Site C.

Site C	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Families	3	3	3	3	3	3	3	3	3	3	3	3
Individuals	7	11	11	18	21	26	34	31	22	35	26	10
Dominance_D	0.35	0.34	0.36	0.36	0.37	0.33	0.36	0.35	0.39	0.43	0.46	0.54
Simpson_1-D	0.65	0.66	0.64	0.64	0.63	0.67	0.64	0.65	0.61	0.57	0.54	0.46
Shannon_H	1.08	1.09	1.07	1.06	1.05	1.10	1.05	1.07	1.00	0.96	0.93	0.80
Evenness_e^H/S	0.98	0.99	0.97	0.96	0.96	1.00	0.96	0.97	0.90	0.87	0.84	0.74

Table 5: Comparison of diversity indices of three sites.

	Site A	Site B	Site C
Families	4	7	3
Individuals	999	1881	252
Dominance_D	0.30	0.18	0.34
Simpson_1-D	0.70	0.82	0.66
Shannon_H	1.30	1.81	1.09
Evenness_e^H/S	0.92	0.87	1.00

Table 6: Similarity index for three sites.

Sample 1	Sample 2	Bray-Curtis index
Site A	Site B	0.693
Site A	Site C	0.120
Site B	Site C	0.070

dominance. Beetles belonging to this site are water beetles and dominate the wetland area thus hindering other beetle families from flourishing. Thus Site C has a different beetle composition from the other two sites. This is also shown in the similarity index and cluster analyses. The only common family of these three sites is Carabidae. The only possible and reasonable conclusion will be that Carabids can explore different habitats. Site B has the highest diversity. It is a residential area and was thought to harbor the least number of beetles, but results obtained were just the opposite. Though being an area of concrete jungle, each and every house has a large expanse of gardens with diverse floral composition, that is, varieties of trees, shrubs, and bushes providing diverse habitat that can support a large variety of beetles.

Though the beetle varieties seen in Site A and Site B are more or less similar, diversity indices are high in Site B. This can be explained as Site A has a fixed floral range, mostly Shal trees (*Shorea robusta*) that can support selective variety of beetles only.

Due to expansion of urban areas, more sites that protect biodiversity are required. This study suggests that an industrial town with high pollution threats, Durgapur, can nonetheless harbor a large number of beetles. Keeping this in mind, further studies can be conducted at other industrial sites for different floral and faunal groups.

Conflict of Interests

The author declares that there is no conflict of interests.

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Research Article

Notes on the Biology of the Cixiid Planthopper *Cixius meridionalis* (Hemiptera: Fulgoroidea)

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With the exception of a handful of economically important species, the biology of cixiid planthoppers (Hemiptera: Fulgoroidea: Cixiidae) is poorly known. The host plants and life history of *Cixius meridionalis* Beirne were investigated in a wetland in Soldotna, Alaska. Specimens were collected over the course of the growing season by hand, aspirator, Berlese funnel, and sweep net. A handful of live nymphs were placed in a terrarium containing potential host plants for direct observation of feeding. *C. meridionalis* was found to feed on roots of *Picea mariana* (Mill.) Britton, Sterns & Poggenb, *Empetrum nigrum* L., *Chamaedaphne calyculata* (L.) Moench, and *Vaccinium vitis-idaea* L. At least within the study area, *C. meridionalis* appears to require multiple years to reach adulthood, with overwintering in nymphal instars. *C. meridionalis* was occasionally tended by *Myrmica alaskensis* Wheeler.

1. Introduction

With the exception of a handful of economically important species that transmit phytoplasmas [1–4], the biology of cixiid planthoppers (Hemiptera: Fulgoroidea: Cixiidae) is largely unknown. Nymphs are subterranean, feeding on plant roots or fungi [3]. Adults generally live aboveground but have been collected underground [2, 3]. Some species have a completely cavernicolous life cycle, with adults feeding on roots along with nymphs [5, 6]. Cixiids are usually univoltine, with diapause in nymphal instars [3], although at least one species requires two years to complete its life cycle [7]. Cixiids are sometimes associated with ants [3, 8, 9], and at least one species appears to be an obligate guest of ants [10].

Cixius meridionalis Beirne is the most widespread and frequently collected cixiid in Alaska based on material in the collection of the University of Alaska Museum (Fairbanks, Alaska) and is broadly distributed in northern North America [11]. No biological data are available for this species other than localities and collection dates of adults [11]. Other *Cixius* species feed on roots of vascular plants (Table 1).

I first observed *C. meridionalis* as adults swept from low vegetation at Headquarters Lake, Soldotna, Alaska, on October 20, 2006. In 2008, I observed cixiid nymphs in a Berlese sample from a wetland near Headquarters Lake (KNWR:Ento:8917, http://dx.doi.org/10.7299/X72807QZ) and in a sample of *Hylocomium splendens* (Hedw.) Schimp. moss from a black spruce forest in the vicinity of the Chickaloon River near Chickaloon Flats. Over the summer of 2013, I sought to determine the host plants and life history of *C. meridionalis*.

2. Materials and Methods

2.1. Field Sampling. Adult and nymphal cixiids were sampled by hand, aspirator, and sweep net in the wetland around Headquarters Lake, Soldotna, Alaska (N 60°27′35″ W 151°03′58″), at least weekly from May 28, when soils in the muskeg were still partly frozen, until the first fall frost on September 20, 2013. Moss samples were collected at least weekly from July 31, 2013, to September 20, 2013, and cixiids were extracted with a BioQuip model 2831 Berlese funnel. Nymphs of *C. meridionalis* were mainly collected by carefully pulling apart moss and duff by hand and extracting nymphs with a BioQuip model 1135A aspirator. Feeding behavior was difficult to observe in the field because nymphs quickly hopped away or crept down into the substrate to avoid capture, but nymphs were occasionally found in dense moss or duff where they could not easily move away from their

Table 1: Known nymphal hosts of *Cixius* species.

Cixius species	Nymphal host	Reference
C. pallipes Fieber	Roots (species unknown)	Vadell and Hoch [6]
C. pilosus (Olivier)	Grasses	China [12]
C. wagneri China	*Fragaria* × *ananassa* (Weston) Duchesne ex Rozier (pro sp.) (*chiloensis* × *virginiana*)	Salar et al. [4]

feeding sites. Roots were identified to species by excavating them back to the aboveground parts of the plants. Infested roots were brought to the laboratory and feeding sites were examined for evidence of feeding.

2.2. Rearing and Identification. Adults were reared from fifth-instar nymphs by placing nymphs in vials or plastic bags with damp *Sphagnum* moss, and then adults were identified using the key of Kramer [11].

To identify the nymphal instars of *C. meridionalis*, I developed a key through examination of the nymphs collected and by comparison with existing descriptions and keys [2, 13, 14].

2.3. Direct Observation of Feeding. In an attempt to observe feeding, several *C. meridionalis* nymphs were placed in a clear plastic terrarium (27 cm length × 17 cm width × 17 cm depth) filled with a correspondingly sized divot of moss and plants from the Headquarters Lake wetland including *Sphagnum* moss, a *Picea mariana* (Mill.) Britton, Sterns & Poggenb (Pinaceae) seedling, *Empetrum nigrum* L. (Ericaceae), *Ledum palustre* L. (Ericaceae), *Vaccinium vitis-idaea* L. (Ericaceae), *Andromeda polifolia* L. (Ericaceae), *Vaccinium oxycoccos* L., *Rubus chamaemorus* L. (Rosaceae), and *Drosera rotundifolia* L. (Droseraceae). The moss was kept moist. Roots visible on the sides and bottom of the terrarium were checked often for feeding activity.

2.4. Specimen Records and Specimen Deposition. Specimens were deposited in the entomology collection of the Kenai National Wildlife Refuge, Soldotna, Alaska (KNWR). Most specimens will be transferred to the entomology collection of the University of Alaska Museum (UAM), Fairbanks, Alaska. Specimen records for both collections are available via Arctos (http://arctos.database.museum/). Complete specimen records for specimens used in this study can be found in Supplementary Table 1 available online at http://dx.doi.org/10.1155/2014/769021.

3. Results

3.1. Habitat and Feeding Behavior. Nymphs were often closely associated with roots of *P. mariana*, but they also appeared to be on roots of *E. nigrum*, *V. vitis-idaea*, and *Chamaedaphne calyculata* (L.) Moench (Ericaceae) in black spruce muskeg. No nymphs were found farther than about 2 m from boles of black spruce trees, even where the microhabitat contained similar moss communities and ericaceous shrubs.

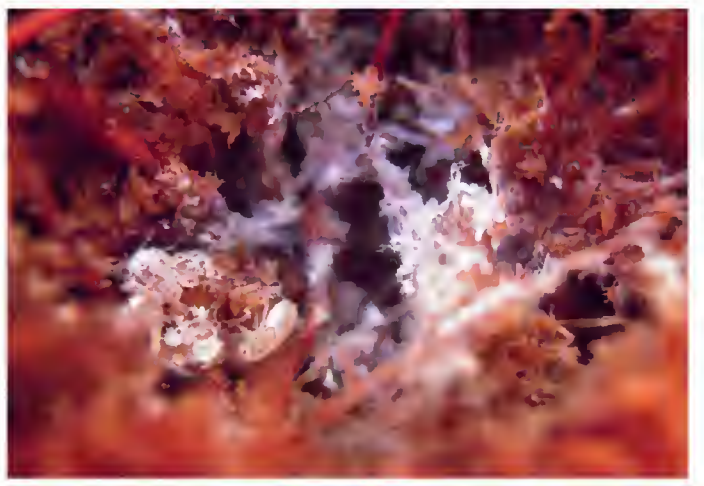

Figure 1: Fourth instar *C. meridionalis* nymph *in situ*, July 12, 2013. The disturbed nymph had moved from its feeding site on a root of *Chamaedaphne calyculata*.

Nymphs were found exclusively in loose, moist moss and duff, where they could move relatively freely through voids (Figure 1). In some places, there was evidence of abundant activity of *C. meridionalis* nymphs judging from the quantities of nymphs collected, copious amounts of waxy secretions, and nymphal exuviae, but it was often difficult to distinguish between feeding sites and waxy secretions of cixiids and other Hemiptera including eriosomatines (Aphididae) and ortheziids (Coccoidea). Feedings sites of cixiids were generally less conspicuous than those of eriosomatines and ortheziids, with sparser waxy secretions and imperceptible damage to roots.

One nymph kept in the terrarium fed on a root of *E. nigrum* (Figure 2). No other feeding was observed in the terrarium.

3.2. Life History. Sampling by all methods yielded 288 specimens of *C. meridionalis* (Figure 3).

Despite targeted searching, only one egg was found that was thought to belong to *C. meridionalis*. This had been in moss inadvertently taken with nymphs on September 4. Nymphs were collected at Headquarters Lake over the entire sampling season. Only three first-instar nymphs were found between June 7 and August 7. Second-instar nymphs were found from June 7 to September 12 and third-instar nymphs were found from June 5 to September 20. Fourth-instar and fifth-instar nymphs were found for the entire portion of the season sampled (May 28 to September 20). My sampling methods appeared to be biased, yielding more large, late-instar nymphs than early instars.

Adult males appeared by July 8 and were observed until September 4. Adult females were found from July 12 to August 27.

Figure 2: Fifth instar *C. meridionalis* nymph feeding on a root of *Empetrum nigrum* in a terrarium.

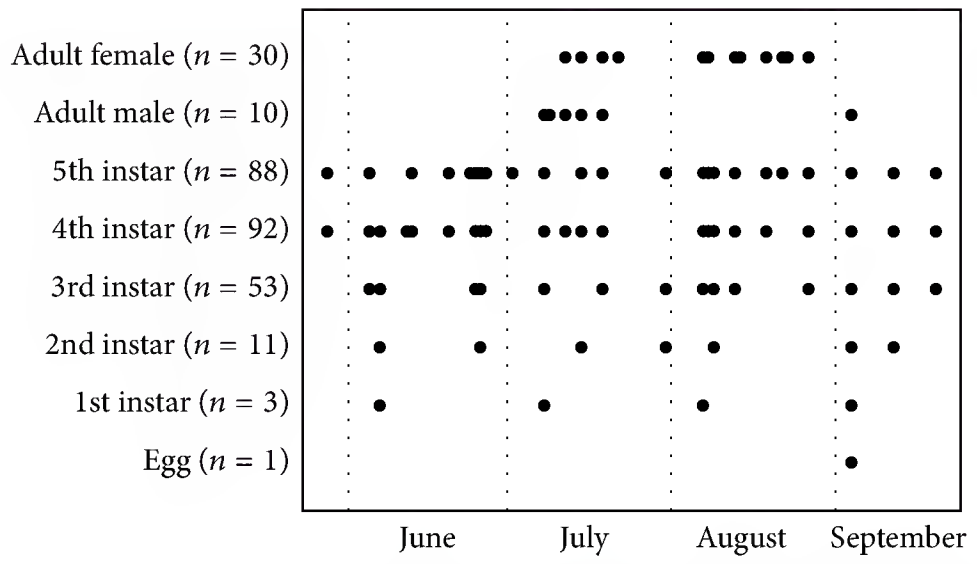

Figure 3: Distribution of life history stages of 288 specimens of *C. meridionalis* collected at Headquarters Lake from May 28 through September 20, 2013.

3.3. Key to the Nymphal Instars of Cixius meridionalis

(1) Metatarsi 3-segmented: mesonotum with a longitudinal row of 2–4 pits near anteromedial corner (2);

Metatarsi 2-segmented: mesonotum with 0-1 pits in anteromedial corner (3);

(2) Mesonotal wingpads extending nearly to apex of metanotal wingpads; mesonotum with a longitudinal row of 2–4 (usually 3-4) pits near anteromedial corner (fifth instar);

Mesonotal wingpads not approaching apex of metanotal wingpads; mesonotum with a longitudinal row of 1-2 (usually 2) pits near anteromedial corner (fourth instar);

(3) Mesonotum with 0-1 (usually 1) pits in anteromedial corner, length 2.1-2.2 mm (third instar);

Mesonotum with no pits in anteromedial corner, length 1.7 mm or less (4);

(4) Abdominal wax plates clearly evident, length 1.6-1.7 mm (second instar);

Abdominal wax plates not apparent, length about 1.2 mm (first instar).

3.4. Additional Observations. None of the nymphs collected were obviously parasitized.

C. meridionalis nymphs, although seldom associated with ants, were not averse to close proximity. When a large colony of *Formica aserva* Forel (including slaves of *Formica neorufibarbis* Emery) in a peaty hummock was excavated, *C. meridionalis* nymphs were found within 2-3 cm of ant tunnels. There was no obvious communication between the ants' tunnels and the feeding places of the nymphs. In one case, a *Myrmica alaskensis* Wheeler worker appeared to be attending a *C. meridionalis* nymph. In another case, *M. alaskensis* workers were found in the same wax-lined cavity with *C. meridionalis* nymphs.

When disturbed, the nymphs would forcibly squirt honeydew from their posterior ends, sending a narrow stream up to about 1 cm away. This was not obvious in the field but was observed when handling live nymphs under magnification.

4. Discussion

Within the area sampled, *C. meridionalis* nymphs appear to be polyphagous on roots of *P. mariana* and ericaceous dwarf shrubs in moist moss and duff. However, the species' consistent proximity to *P. mariana* suggests that it is either dependent on *P. mariana* at some stage in its life history or the two species share similar microhabitat requirements. In addition to the wetland habitat at Headquarters Lake, *C. meridionalis* has been collected from moist *Sphagnum* moss in well-drained black spruce forest in Kasilof, Alaska (KNWR:Ento:8918, http://dx.doi.org/10.7299/X7XG9R8K).

Phytoplasmas were recently reported from Poland in *Picea abies* (L.) Karst. and the two imported Nearctic species *Picea glauca* (Moench) Voss and *Picea pungens* Engelm. [15]; none are currently known from *P. mariana*. As potential vectors, it would be interesting to check for the presence of phytoplasmas in cixiids associated with *Picea* spp., especially *Cixius beieri* Wagner, whose adult hosts include *Picea* in central Europe [16].

Based on the observed phenology at Headquarters Lake, the life history of *C. meridionalis* is suggested to be as follows. Eggs are deposited in the late summer, hatching in the fall or spring. The nymphs appear to take multiple years to reach adulthood, overwintering in place as nymphs. Adults emerge in early July and are active until late September. However, adults can be found over a longer season. The University of Alaska Museum has adults collected as early as June 23 from interior Alaska (UAM:Ento:176723) and I have observed adults at Headquarters Lake as late as October 20.

The unusually long nymphal stage of *C. meridionalis* at Headquarters Lake as compared to most other cixiids may be due to the cold soil temperature of this wetland, a protracted

life cycle being a common adaptation of insects to cold climates [17].

As with some other cixiids, *C. meridionalis* is at least occasionally tended by ants, but the association is facultative and infrequent, fitting best into the "opportunistic and occasional" category of ant attendance defined by Bourgoin [18].

This species' lack of spines on the fore tibiae, common on many cixiid nymphs, may be an adaptation to the loose, mossy microhabitat in which it lives, where such spines would not be necessary for digging.

Conflict of Interests

The author declares that there is no conflict of interests regarding publication of this paper.

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Research Article

An Ultrastructural and Fluorescent Study of the Teratocytes of *Microctonus aethiopoides* Loan (Hymenoptera: Braconidae) from the Hemocoel of Host Alfalfa Weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae)

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The braconid wasp *Microctonus aethiopoides* Loan is an idiobiont endoparasitoid of alfalfa weevil adults *Hypera postica* (Gyllenhal). After oviposition and subsequent egg maturation, large trophic cells called teratocytes dissociate from the serosa and are released into the host hemocoel. These teratocytes are present in large numbers and are visible to the naked eye. It is thought that they accumulate host hemocoelic metabolites for later consumption by the parasitoid larvae. We have undertaken a microscopic study of these gargantuan and complex cells at approximately seven months after parasitization. Parasitized adult weevils were dissected into medium and teratocytes were fixed, embedded, and sectioned at 1 μm. Teratocytes were stained with various specific fluorescent dyes for plasma membrane, Golgi, nuclei, lysosomes, mitochondria, and endoplasmic reticulum (ER). The surface of each cell is covered with a dense microvillar layer. Analysis of fluorescent images showed that these cells do not have condensed nuclei. ER was abundant around the nuclear envelope. Lysosomes were positioned around the periphery of the nucleus and the Golgi apparatus was significantly enlarged, being located around the nuclear envelope.

1. Introduction

Microctonus aethiopoides Loan (Hymenoptera: Braconidae) was found to be an effective biological control agent for the adult alfalfa weevil *Hypera postica* (Gyllenhal) [1, 2]. *M. aethiopoides* is an idiobiont parasitoid which prevents further host development after parasitization. Following oviposition into the hemocoel of the adult alfalfa weevil host the *M. aethiopoides* egg hatches, releasing both a first instar parasitoid larva and numerous free floating extraembryonic serosal cells which subsequently develop and differentiate into teratocytes [3]. Teratocytes undergo significant hypertrophic growth within the hemocoel of the host. The presence of these large, white opaque cells has been implicated in the determination of host range of *M. aethiopoides* [4–6].

Teratocytes or teratocyte-like cells have been documented in developmental studies of four hymenopteran families: Braconidae, Ichneumonidae, and Platygastridae [7, 8] and have been reported in a single species of chalcids [9]. Teratocytes are thought to function primarily as trophic cells which assimilate host metabolites for later ingestion by the developing parasitoid larvae [7]. However, teratocytes secrete a number of proteins *in vivo* and *in vitro* which may be responsible for the observed alterations of the host endocrine system [10–15] and suppression of the host's immune response against the parasitoid egg and larvae [16–18]. Despite their importance to successful parasitization by braconid parasitoids and their unique functions in suppression of the host immune system and alterations of host endocrinology, the ultrastructure of

teratocytes has been rarely studied [9, 19–21]. The present study was undertaken to elucidate and to document the ultrastructure of these extremely large and important cells.

2. Materials and Methods

2.1. Insects and Teratocyte Collection. Nondiapausing alfalfa weevils, *Hypera postica* parasitized by *Microctonus aethiopoides,* used in this study were obtained by sweeping adults from an alfalfa field near Boonville, Missouri (Cooper Co.) on April 29, 2002. These weevils were collected in late spring and early summer. The weevils were returned to the laboratory and maintained at 5°C on a bouquet of alfalfa in a 0.275 L ice cream carton with a Petri dish as a lid until May 1, at which time some were initially dissected under a dissecting microscope in a Stender dish cover in distilled water to assess the extent of parasitization. Adult weevils were grasped with forceps and a needle inserted in anus and gently teased to expose the contents of the abdomen. A careful search was made to locate the parasite larvae to confirm parasitization. If the host was parasitized teratocytes floated into the dissection medium. The identity of the parasite larvae as *M. aethiopoides* was confirmed by emergence of adult parasites reared from adult weevils collected from the same site. Dissections from weevils maintained in the same manner but for fixing, staining and sectioning of teratocytes Excel 401 tissue culture medium (Gibco Invitrogen; Carlsbad, CA) was used as dissection medium. Dissections were completed 2 days later (May 3), and again on May 6, May 8, and May 10. Teratocytes were collected from the medium for processing as detailed below.

2.2. Light Microscopy. Teratocytes were immediately fixed in 3% paraformaldehyde in HEPES wash buffer for 10 min in a gentle vacuum and 2 hrs at room temperature for fluorescent studies. For light microscopy and TEM studies, the teratocytes were placed in fixative solution (2.5% glutaraldehyde/2% paraformaldehyde, 70 mM HEPES, and pH 7.4) for 3 hrs. Then, the teratocytes were dehydrated in an ethanol series, infiltrated, and embedded in methacrylate for fluorescent studies and in Epon/Spurr's resin for light microscopy. Semithin sections of the teratocytes were stained with 0.5% acid fuchsin/0.5% toluidine blue in ultrapure water. The slides were air-dried at room temperature, mounted with Permount, and viewed with an Olympus (Melville, NY) or Nikon (Melville, NY) microscope. Images were captured using ImagePro (Media Cybernetic, Silver Spring, MD) and Spot (Diagnostic Instruments, Inc.) software and further edited using Adobe Photoshop 6.0 software.

2.3. Fluorescence Microscopy. Using a modification of the technique of Baskin et al. [22–24] teratocytes were encased in a sandwich of Formvar supported on a copper wire loop. A copper wire loop (36 ga) was made and flattened between two flat pieces of steel. Small rectangular films of 0.25% of Formvar in ethylene chloride were floated on water and the loop plunged into the middle of rectangle so that a film of Formvar surrounded the wire. A number of loops were made in advance. The loops covered with a film of Formvar were placed on a drop of water on a piece of clean glass separately. The fixed teratocytes were gently placed on the center of the Formvar surface. This assembly was coated with another layer of Formvar, sandwiching teratocytes between two layers of Formvar.

Teratocytes sandwiched between layers of Formvar were dehydrated in an ethanol series at −20°C for 30 minutes for each step and then infiltrated with methacrylate (80% butyl methacrylate, 20% methyl methacrylate, and 0.5% benzoin ethyl ether; Aldrich) and 10 mM DTT. Teratocytes were embedded in fresh methacrylate mix in BEEM capsules and the plastic was polymerized under UV light in a cold room (4°C) overnight. Blocks were sectioned at 1 μm using a Reichert Cut S ultramicrotome (Leica, Wien, Austria). Serial sections of teratocytes were placed on drops of 5% ammonium hydroxide on silane-coated slides. The sections were annealed onto the slide by gentle heating on a slide warmer and the slides were stored at 4°C for further staining. Entire 1 μm sections of eight teratocytes were deplasticized with acetone for 10 minutes, washed with HEPES buffer for 30 minutes, and cleared with 0.1% Tween 20 in PBS for 15 minutes. Nonspecific binding was minimized by incubation in blocking buffer (5% BSA, 1% nonfat dry milk, 1% gelatin, and 0.01% sodium azide).

Organelle specific stains purchased from Molecular Probes (Eugene, OR) were incubated with semithin sections according to manufacturer's recommendations (http://www.probes.com). Initial range finding experiments with fluorescent dyes, over several orders of magnitude, allowed us to derive an optimal concentration for each fluorescent dye. The sections of two cells were stained with 500 nM of Dil (a plasma membrane specific stain) in HEPES wash buffer for 90 min. Then the sections were washed with the same buffer for 30 min and were counterstained with 500 nM TO-Pro3 (a DNA specific stain) for 90 min. Sections of two other teratocytes were stained with 2 μM of $NBDC_6$ (a Golgi apparatus specific stain) for 90 min and counterstained with 100 nM of Lysotracker (a lysozyme specific stain) for 90 min. The sections of two more teratocytes were stained with 250 nM of Mitotracker (a mitochondrial specific stain) for 90 min and counterstained with 500 nM of $DioC_6$ (an endoplasmic reticulum specific stain) for 90 min. Slides were mounted with Moviol and were imaged by confocal microscopy.

2.4. Transmission Electron Microscopy (TEM). Teratocytes encased between two Formvar layers were fixed in 2% glutaraldehyde/2% paraformaldehyde in 0.1 M cacodylate buffer. After fixation, the samples were rinsed three times in buffer and then postfixed in 1% OsO_4 in the same buffer. Samples were then rinsed three times for 20 min each with ultrapure water. Tertiary fixation was done in 1% aqueous uranyl acetate, followed by three rinses of 20 min each in ultrapure water. The samples were dehydrated in an ethanol series and infiltrated with Epon/Spurr's resin, and the resin was then polymerized at 55°C for two days after which the blocks were stored in a desiccator until sectioning. Ultrathin sections were

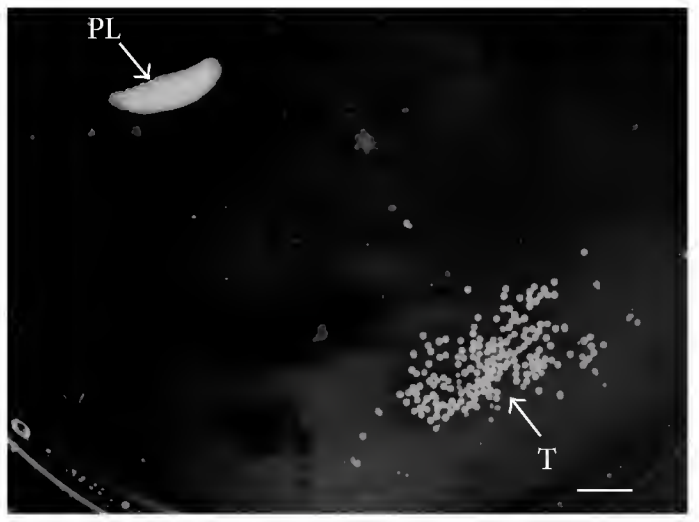
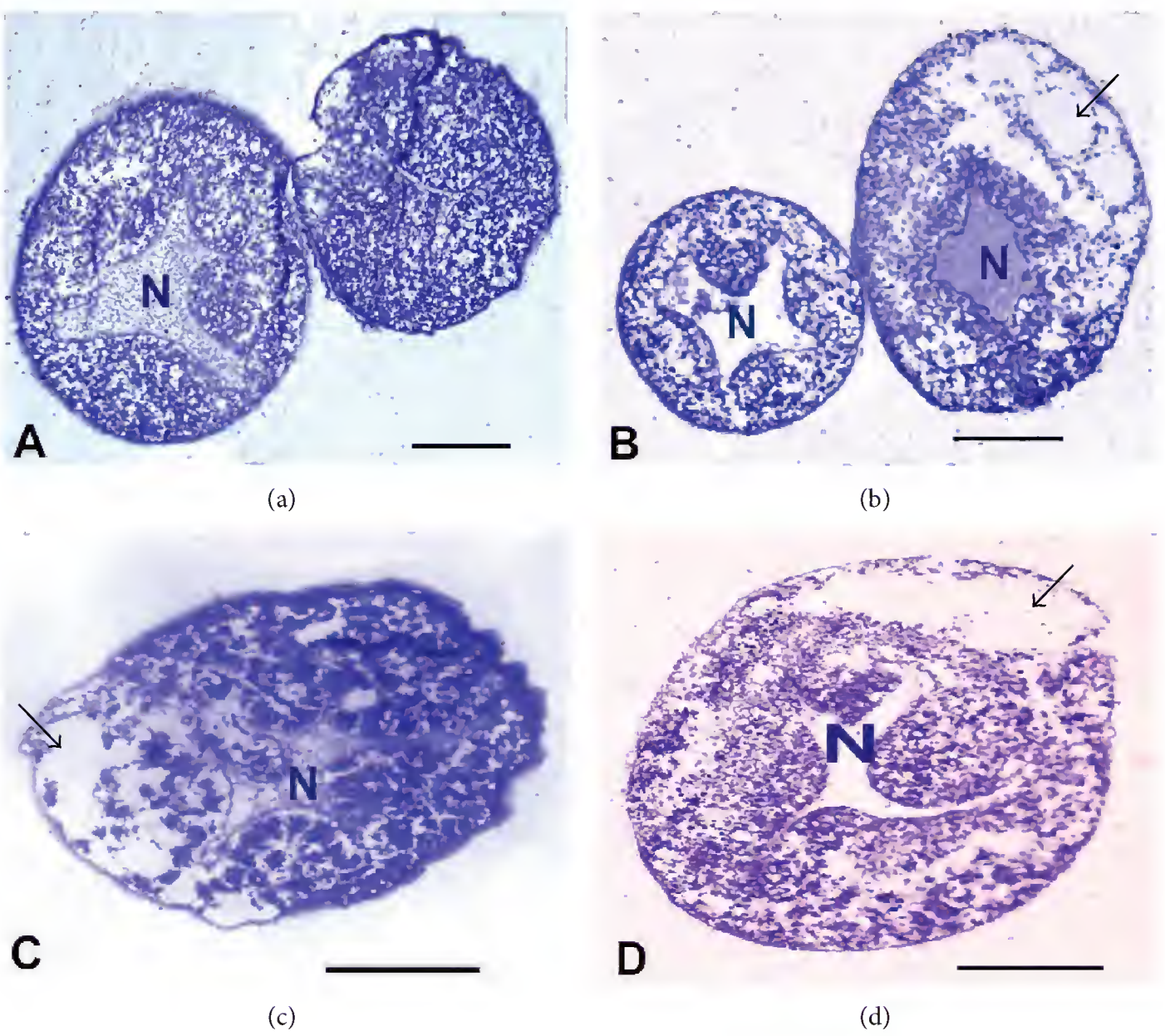


FIGURE 1: Bright-field image of *M. aethiopoides* larva and teratocytes collected from a single host adult *H. postica* hemocoel at approximately seven months after parasitization showing hypertrophied *M. aethiopoides* teratocytes. PL: parasitoid larvae, T: teratocytes, scale bar = 1 mm.



(a) (b)

(c) (d)

FIGURE 2: Bright-field images of different 1 μm (semithin) sections from different areas of teratocytes of *M. aethiopoides* were stained with 0.5% toluidine blue and counterstained with 0.5% acid fuchsin showing amorphous nuclei. N: nuclei. Large unstained areas (arrow). Scale bar = 50 μm.

cut with a Reichert Ultracut S ultramicrotome (Leica, Wien, Austria). Standard TEM procedures were applied to ultrathin sections of the teratocytes on copper grids.

3. Results

Teratocytes collected from the hemolymph of field-collected adult alfalfa weevils at approximately seven months after parasitization were examined by bright-field microscopy. When parasitized weevils were dissected under saline or tissue culture medium numerous teratocytes were easily visualized. No attempt was made to definitively determine the mean number of teratocytes per host. Teratocytes were extremely large hypertrophied, opaque buoyant spherical cells, visible to the naked eye (Figure 1). Since only a single developmental stage was available for study, earlier stages of teratocyte development, release, and growth were not observed and are not presented in this study. At the time of collection, all teratocytes appeared to have attained maximal diameter. A cytological investigation of teratocyte internal structures

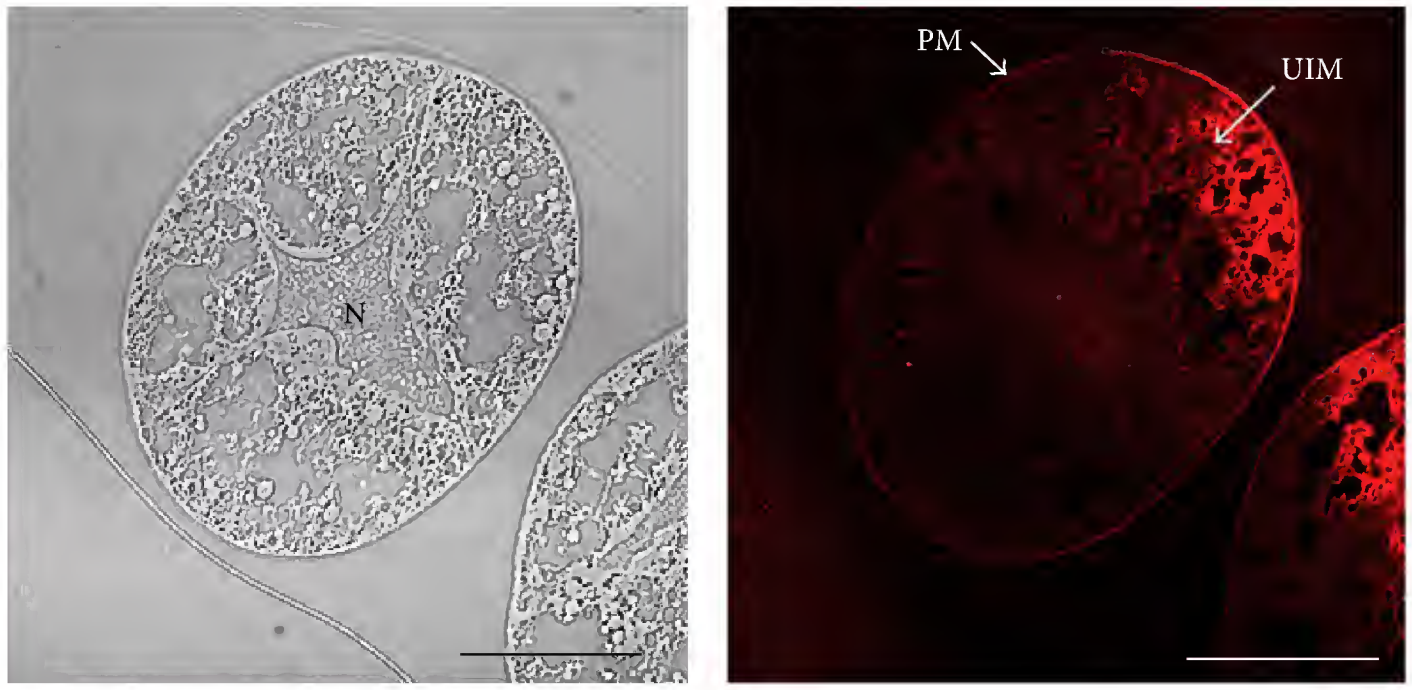


FIGURE 3: Semithin sections of teratocytes were dual-stained with Dil for plasma membrane and with TO-Pro3 for nucleus. (a) Transmitted light micrograph of teratocyte showing internal structure of the cell. (b) Same cell section stained with Dil showing strong staining of plasma membrane and an unidentified internal membranous network. (c) Same section stained with TO-Pro3 showing intense staining of chromosomal DNA within the large amorphous nucleus. (d) Overlaid images from (a) to (c) showing red plasma membrane and blue amorphous nucleus. N: nucleus, PM: plasma membrane, UIM: unidentified internal plasma membrane network. Scale bars = 20 μm.

was undertaken based on light and fluorescent microscopic examination of a large number of semithin sections.

Semithin sections were stained with toluidine blue/acid fuchsin to obtain resolution of intracellular structures (Figures 2(a)–2(d)). A large stellate nucleus occupied the center of each teratocyte, with ramifications extending throughout the cytoplasm to the plasma membrane. The remainder of the interior was occupied by densely staining granules. In all teratocytes examined, a curious unstained internal membranous network at one end of each teratocyte and occupying a substantial volume was present in close proximity to the plasma membrane (Figures 2(b)–2(d)).

Further examination of teratocyte intracellular structure was undertaken by fluorescence microscopy of semithin sections using dyes chosen for their ability to stain specific organelles (Figures 3(a)–3(d)). Sections were dual-stained with the plasma membrane specific stain, Dil, and with the nuclear stain TO-Pro3. A wide plasma membrane surrounding each cell was observed, and a polar structure comprised of an internal membranous network heavily stained by Dil was present at the periphery of each teratocyte examined (Figure 3(b)). The same sections stained with TO-Pro3 exhibited intense staining of chromosomal DNA within the large stellate nucleus (Figure 3(c)). The nucleus did not appear to overlap with the unidentified internal membranous network (Figure 3(d)).

Semithin sections were dual-stained with the Golgi specific stain $NBDC_6$ and with the lysosomal specific stain Lysotracker (Figures 4(a)–4(d)). Within each teratocyte multiple strongly staining Golgi structures were observed distributed along the periphery of the nucleus (Figure 4(b)). Numerous lysosomal-staining structures were distributed throughout the volume of the teratocyte (Figure 4(c)). When the images were digitally overlaid, the perinuclear position of

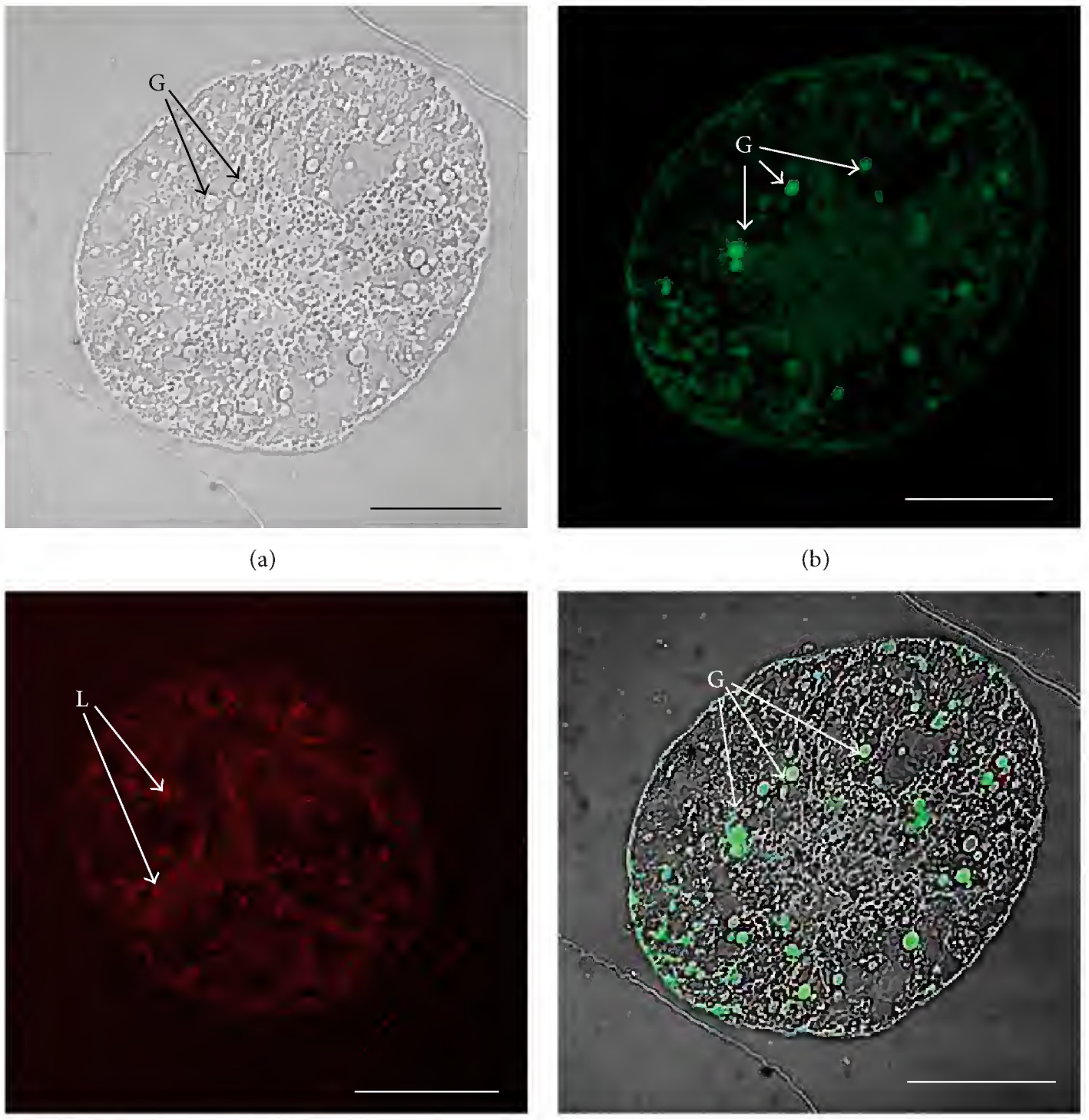


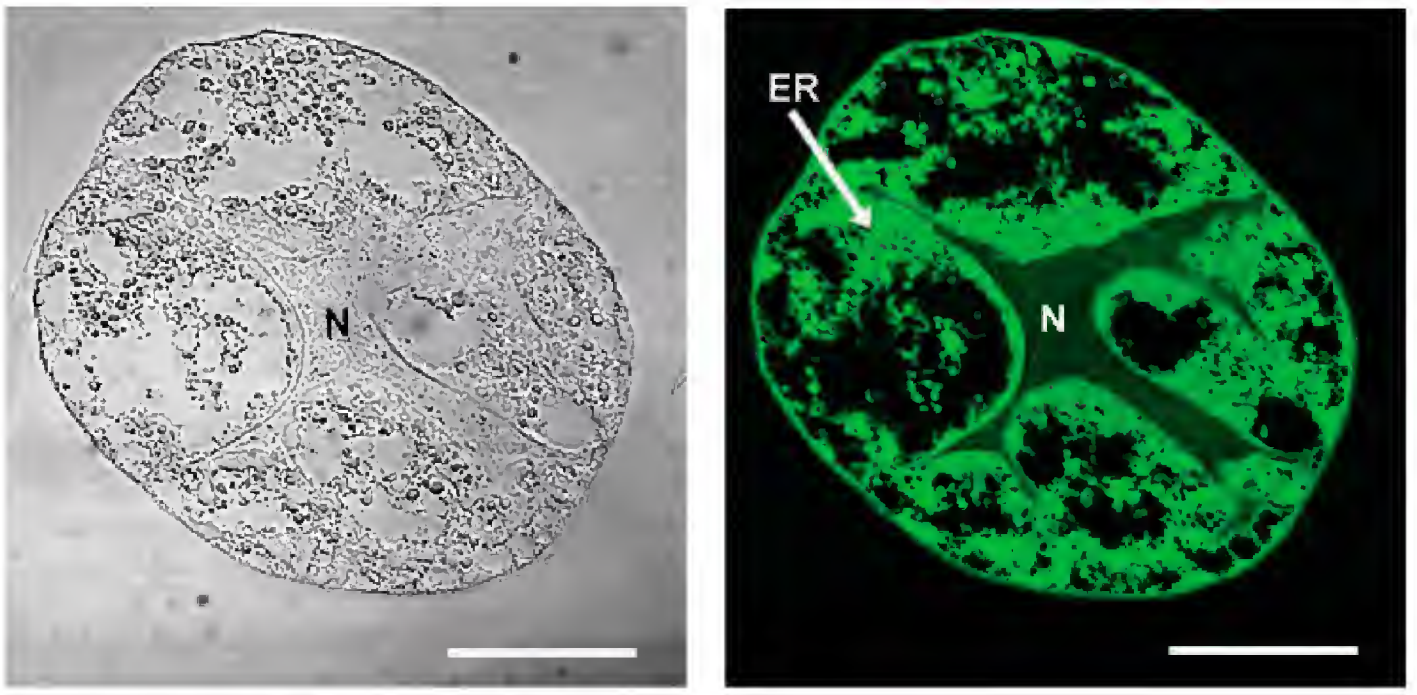
FIGURE 4: Semithin sections of teratocytes were dual-stained with $NBDC_6$ for Golgi apparatus and Lysotracker for lysosomes. (a) Transmitted light micrograph of teratocyte showing internal structure of the cell. (b) Same cell section stained with $NBDC_6$ showing strong staining of the Golgi distributed mostly around the periphery of the nucleus. (c) Same section stained with Lysotracker showing distribution of lysosomes (red) within the teratocyte. (d) Overlaid images of (a)–(c) showing Golgi around nucleus. G: Golgi apparatus, L: lysosomes. Scale bars = 20 μm.

the multiple Golgi was observed (Figure 4(d)). Sections dual-stained with the mitochondrial specific dye Mitotracker and the ER specific dye $DiOC_6$ revealed the close proximity of protein synthesis with energy production (Figure 5(a)). Dark unstained regions were seen within lobes of the teratocyte bordered by intense ER staining (Figure 5(b)). Mitochondria are present throughout the same volume of teratocyte occupied by intensely staining ER (Figure 5(c)).

Ultrastructural studies were undertaken to obtain a more detailed view of the highly complex teratocyte cytoplasm. The plasma membrane appeared to be composed of a dense lawn of microvilli. Substantial amounts of rough ER cisternae and mitochondria were closely associated with plasma membrane in close proximity to the microvilli (Figures 6(a) and 6(d)). The membranous network located at one end of the teratocytes was composed of highly complex and folded membranes with a tubular appearance that could perhaps be invaginations of the plasma membrane involved in uptake of materials from the host hemocoel (Figure 6(b)). The function of this structure has yet to be determined. The stellate nucleus was surrounded by rough ER, Golgi (Figure 6(c)), and lipid droplets (Figure 6(d)). Higher magnification revealed mitochondria, lipid droplets, and darkly staining granules throughout the cytoplasm between the nucleus and the plasma membrane (Figure 6(e)). The internal structure of the same area of the teratocyte showed large numbers of starch and pigment granules and of lipid droplets (Figure 6(f)).

4. Discussion

Teratocytes collected from the hemolymph of field-collected adult alfalfa weevils at approximately seven months after parasitization were examined by bright-field microscopy. When parasitized weevils were dissected under saline or tissue culture medium numerous teratocytes were easily visualized. No attempt was made to determine the mean number of



(a) (b)

(c)

FIGURE 5: Semithin sections of teratocytes were dual-stained with Mitotracker for mitochondria and with $DiOC_6$ for endoplasmic reticulum. (a) Transmitted light micrograph of teratocyte showing internal structure of the cell. (b) Same cell section stained with $DiOC_6$ showing green staining of ER surrounding the dark unstained bodies. (c) Same section stained with Mitotracker (red) showing distribution of mitochondria within the teratocyte. ER: endoplasmic reticulum, M: mitochondria, N: nucleus. Scale bars = 20 μm.

teratocytes per host. In comparison to the developing *M. aethiopoides* larva from the same host, the teratocytes were extremely large hypertrophied, opaque buoyant spherical cells, visible to the naked eye. Since only a single age was available for study, the initial stages of teratocyte release and growth were not observed. At the time of collection, all teratocytes appeared to have attained maximal diameter. No earlier teratocyte developmental stages were examined. An initial cytological investigation of the internal structures was undertaken. Semithin sections of the same material were stained with toluidine blue/acid fuchsin to obtain resolution of intracellular structures. In a manner similar to the teratocytes of *Toxoneuron* (=*Cardiochiles*) *nigriceps* (Viereck) [20], a large stellate nucleus occupied the center of each teratocyte, with ramifications extending throughout the cytoplasm to the plasma membrane. The remainder of the interior was occupied by densely staining granules. An unstained internal membranous network occupying a substantial volume was present in close proximity to the plasma membrane.

Ultrastructural studies were undertaken to obtain a more detailed view of the highly complex teratocyte cytoplasm. The plasma membrane appeared to be composed of a dense lawn of microvilli, similar in appearance to teratocytes of another braconid wasp, *Microplitis croceipes* (Cresson) [19]. Substantial amounts of rough ER cisternae and mitochondria were closely associated with plasma membrane. Close association of the microvilli, rough ER, and mitochondria at the plasma membrane indicates that both uptake of nutrients from and the synthesis/secretion of proteins into the host hemocoel are the primary activities occurring at this stage of teratocyte development [20, 25]. The membranous network located at one end of the teratocytes was composed of highly complex and folded membranes with a tubular appearance that could perhaps be invaginations of the plasma membrane involved in uptake of materials from the host hemocoel. The function of this structure has yet to be determined. The stellate nucleus was surrounded by rough ER, Golgi, and lipid droplets. Higher magnification revealed mitochondria,

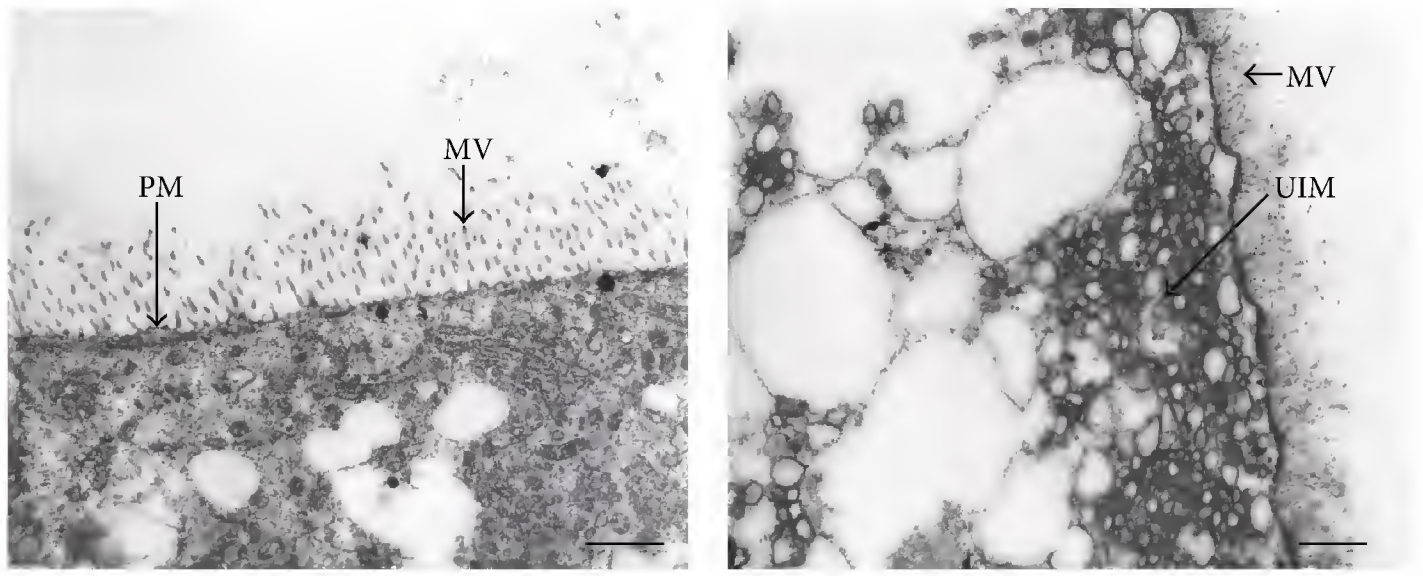


FIGURE 6: Ultrathin sections of teratocytes were stained with uranyl acetate and Lead citrate. (a) Dense microvilli, rough endoplasmic reticulum, and mitochondria associated with plasma membrane. Scale bar = 500 nm. (b) TEM image of unidentified membranous network located at opposite sides of the teratocyte. Scale bar = 1 μm. (c) TEM image of one arm of amorphous nucleus showing intense staining of heterochromatin, numerous lipid droplets, and pigment. Scale bar = 1 μ. (d) Same cell showing microvilli, rough ER, and associated mitochondria. Scale bar = 500 nm. (e) Same cell with higher magnification showing mitochondria and lipid droplets. Scale bar = 100 nm. (f) Electron micrograph of the internal structure of the same area of the teratocyte showing starch and pigment granules and lipid droplets. Scale bar = 500 nm. PM: plasma membrane; MV: microvilli; UIM: unidentified membranous network; N: nucleus; L: lipid droplets; RER: rough endoplasmic reticulum; M: mitochondria; S: starch; P: pigment granules. Scale bar = 20 μm.

lipid droplets, and darkly staining granules throughout the cytoplasm between the nucleus and the plasma membrane. Within this area of the teratocyte adjacent to mitochondria were putative energy storage depots such as lipid droplets and starch granules.

In conclusion, the extremely large *M. aethiopoides* teratocytes possess an internal structure well suited to their putative trophic and secretory function. The plasma membrane is studded with a dense array of microvilli which would facilitate efficient uptake of nutrients from the host hemocoel. Lysosomes, numerous lipid droplets, and granules of protein and starch crowd the cytoplasm. An amorphous membranous network staining heavily with an ER specific dye is present at one end of the cell. Arms of the large stellate

nucleus ramify throughout the volume of the cell abutting against rough ER and mitochondria. Finally, these teratocytes contain large amounts of Golgi, rough ER, and mitochondria that would be required for the synthesis and export of proteins into the host hemocoel that may be responsible for suppression of the host immune system.

Conflict of Interests

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

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Research Article

Inflorescences of the Bromeliad *Vriesea friburgensis* as Nest Sites and Food Resources for Ants and Other Arthropods in Brazil

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For the first time, the usage of bromeliad inflorescences as nesting sites for ants and other arthropods was studied. Frequencies of occurrence of nests were recorded from hollow stems of dried infructescences of the bromeliad *Vriesea friburgensis* on Santa Catarina Island, southern Brazil. Three habitat types were studied: miconietum and two types of restinga, one with low (restinga-low) and one with high vegetation cover (restinga-high). Additionally, flower visitation by ants was examined in restinga-low. Out of 619 infructescences, 33% contained nests. Ants were the most frequent occupants (82–96% of nests), followed by termites (3–18%) and bees (0–0.6%). Species accumulation curves and diversity indices indicate that the diversity of stem-occupying ant species is highest in restinga-low (eight species observed, 18 predicted) and lowest in restinga-high (four observed and predicted). Highest similarity of compositions of infructescence-inhabiting ant species was recorded between miconietum and restinga-high, lowest between restinga-low and restinga-high. Similarity between compositions of inflorescence-visiting and infructescence-inhabiting species in restinga-low was even higher (compared with the cases described in the previous sentence) although 50% of the involved species were present in only one of the samples. Altogether, our results indicate that inflorescences are important resources for ants and other nest-building insects from flowering season to past-fruiting season.

1. Introduction

Bromeliads (Bromeliaceae) are monocot plants occurring almost exclusively in the neotropics [1]. Animal-bromeliad interactions are highly diverse and have been in the focus of intensive research during the last decades [2]. Consequently, several aspects of these associations are well studied, for example, pollinator systems [3–5] or usage of bromeliad rosettes as nest sites by ants [6, 7] and bees [8]. Additionally, bud and fruit capsules are known to nourish and shelter developmental stages of wasps [9] and butterflies [10].

The stalks of bromeliad inflorescences or infructescences have mainly been reported as subjects of insect larval herbivory. For example, inflorescence stems may be infested by several species of Curculionidae (Coleoptera) [2]. In the bromeliad species *Vriesea friburgensis* Mez var. *paludosa* (L. B. Smith) L. B. Smith 1952, flower buds are sterilized by the feeding behavior of a eurytomid wasp larva. Affected flowers do not open and eventually dry up forming a resistant pupal chamber for the developing larva [9]. A similar case is that of *Strymon serapio* (Godman and Salvin 1887) [10] whose larvae attack the ripening fruit capsule of *V. friburgensis*. During feeding, the larva enters the capsule and finally pupates within. In both cases, the imagines emerge from their pupal chambers after weeks or even months. This is enabled by the fact that the stalk of the drying infructescence usually remains standing erect in the rosettes for one year or longer,

instead of wilting and decomposing rapidly (pers. obs.). This feature is typical for many species in the genus *Vriesea* which has an anemogamous seed dispersal syndrome, and allows the small seeds provided with pappi to take flight with the wind. This is in sharp contrast to other bromeliads in the subfamily Bromelioideae whose seeds are embedded into a fleshy pulp and whose infructescence stalks wilt and collapse soon after the colorful berries have been eaten by birds and small mammals.

In the course of a study on the diversity and interactions of flower visitors of bromeliads in the Atlantic rainforest of southern Brazil, we discovered stems of old infructescences of *V. friburgensis* to be inhabited by ant species. This, together with the other mentioned characteristics of the infructescence stalk, suggests that it might play another important role in the ecosystem by providing shelter or nest sites with a beneficial environment for perennial arthropods and social insects. Among the latter, ants constitute the dominant animal group in most terrestrial ecosystems [11], and numerous species that are unspecialized nesters could benefit from the properties of infructescence stems. Therefore, assessing the use of stalks as nesting sites was the main purpose of our study.

Not only infructescences but also inflorescences are, at least during flowering, attractive to ants: in a preliminary census, about 50% of 159 open flowers (distributed over 68 inflorescences) were visited by at least one ant (S. Langner, unpubl. data). The inflorescences are visited by a high diversity of animals, mainly bees [12]. Yet, ants have not been systematically registered so far; hence, an additional goal of our study was to survey the spectrum of ant species associated with inflorescences and infructescences.

In particular, we determined frequencies of occurrence as well as alpha and beta diversity (i.e., diversity within and among habitats) of ants and other arthropods in old infructescence stems of *V. friburgensis*. We expected differences in ant species richness (which is one component of diversity) and composition among habitat types due to different species communities as was reported for inflorescence-visiting ants of other bromeliad species [13]. At one site, we also recorded ants visiting inflorescences for comparison with the stem-inhabiting ants, testing the hypothesis that species richness and composition should be similar because ants living within the bromeliads can be expected to visit nearby flowers.

2. Material and Methods

2.1. Study Plant. *Vriesea friburgensis* is a common, mostly soil-growing but facultatively epiphytic tank bromeliad occurring in forest and restinga habitats in southern Brazil [12, 14, 15]. Its inflorescences, flowering from November to March [9, 12], reach a height of about 0.5–1.5 m (for habitus see Supplementary Figures S1 and S2 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/396095). The dry infructescences frequently remain standing erect in the rosettes for one year or longer, and even when bent or broken, they may stay more or less intact for a long period of time (pers. obs.). Only terrestrially growing plants were examined.

2.2. Study Sites and Period. The study was conducted from January 2006 to January 2009 in the municipality of Florianópolis on Santa Catarina Island, southern Brazil. Infructescence occupation was examined between August and February, inflorescence visitation in December and January. Observations and sampling were performed in the two habitat types "miconietum" (a pre-forest succession stage, [8]) and "restinga" (a xerophilous vegetation formation on sand dunes, [16]). Four sites were studied: (i) a mountainside in Santo Antônio de Lisboa (miconietum; 27°30′26″S, 48°30′28″W); (ii) Joaquina beach (27°37′37″S, 48°26′59″W) (see Supplementary Figure S2) and (iii) Campeche beach (27°40′38″S, 48°28′48″W), both similar low-vegetation restingas and pooled as "restinga-low"; (iv) Reserva Ecológica do Morro das Aranhas (high-vegetation restinga, termed "restinga-high"; 27°28′11″S, 48°23′06″W). An overview of most samples described in the following subsections is provided in Supplementary Figure S3.

2.3. Assessing Infructescence Occupation. We examined a total of 619 infructescences (defined as the stem and remains of buds and fruits above the level of the water reservoir in the rosette) for nests of ants and other social insects by breaking them apart. Criterion for the record of a nest was the presence of brood. Other arthropods were occasionally registered too.

From a subset of 131 infructescences (restinga-low: 54; restinga-high: 28; miconietum: 49), inhabitants of the interior of the stem as well as of cavities under bracts were hand-collected and identified in the laboratory. For the other fraction (488 infructescences, only restinga-low), we identified the inhabitants directly in the field and additionally noted whether the stems were solid or hollow. Cavities under bracts were not examined in this case. To estimate cavity volume, five hollow infructescences were randomly chosen and the lengths and inner diameters (at base and apex) of their cavities were measured.

In addition to the samples described above, we recorded arthropods in infructescences during occasional field trips that were not specifically associated with this study. Sometimes, we also examined the most basal, humid part of the stems. Those findings are separately presented in the results section and Supplementary Table S9 but were not taken into account for the remaining data presentation and analysis. Generally, sets of infructescence stems that served as references for calculation of percentages included those that were not hollow. This is because we regard massive stems to be a resource for ants too, at least for those that are capable of gnawing holes into the plant material themselves.

Three samples of non-social insect brood were taken to the laboratory and reared to adult stages for identification. Voucher specimens of all collected species were deposited in the entomological collection of the Native Bee Laboratory, BEG, Federal University of Santa Catarina, Florianópolis, Santa Catarina, Brazil.

2.4. Inflorescence Visitation by Ants. For identification of ants foraging on inflorescences, specimens were hand-collected from a set of 33 flowering inflorescences (from flower-bearing branches or the nearby stalk; see Supplementary Figure S1) at Joaquina. In two cases, ants were present but could not be identified because they escaped collection. Moreover, the presence of ants within flowers was recorded at the same site, where a further sample of 101 randomly chosen inflorescences was defined. On five days in weekly intervals, all available open flowers on these inflorescences were examined for ants. Because inflorescence lifetime of *V. friburgensis* usually includes days without open flowers [12], the number of inflorescences with open flowers varied between 40 and 79 (out of 101).

For investigating whether the attractiveness of inflorescences begins with the emergence of open flowers or earlier, we assessed ant presence for a separate set of 26 inflorescences with buds only.

2.5. Species Richness and Diversity. We compared the diversity of ants occupying infructescences among habitats (three comparisons) as well as infructescence occupation with inflorescence visitation in restinga-low. Alpha diversity (diversity within each locality) was assessed by computing species accumulation curves (generated with the "Mao Tau" binomial mixture model by Colwell et al. [17]), the Chao2 species richness estimator, and the reciprocal Simpson index. Since Simpson diversity is a measure that combines species richness with the evenness of the species abundance distribution, we also calculated Simpson evenness by dividing the Simpson index by the number of observed species (as given by the species accumulation curves) [18]. Accumulation curves and means (obtained by 1000-fold resampling) of estimators and indices were plotted against the cumulative number of species occurrences as a measure of sampling effort [19]. If such a curve reached a plateau over a logarithmically scaled x-axis, we regarded the corresponding index or species richness as stable [19]; if not, then it was expected to change with increased sampling.

For evaluating beta diversity (i.e., complementarity between sites, [18]) of ant species inhabiting infructescences and visiting inflorescences among the three habitats we calculated Chao's estimator for the Jaccard similarity index ("Chao-Jaccard") with 95% confidence intervals for statistical comparisons. Since similarity is reciprocally related to beta diversity [18], a low similarity index indicates high beta diversity among the sites compared. All species richness and diversity computations were performed with the software package EstimateS 8.2 [20].

3. Results

3.1. Infructescence Occupation. Overall, 205 of all 619 examined infructescences (33%) were occupied by nests of ants (Supplementary Figure S4), termites, or bees. Depending on habitat, ant nests were found in at least 18% of the stems (Figure 1) and made up the majority of nesting occupants (miconietum: 82%; restinga-low: 97%; restinga-high: 94%).

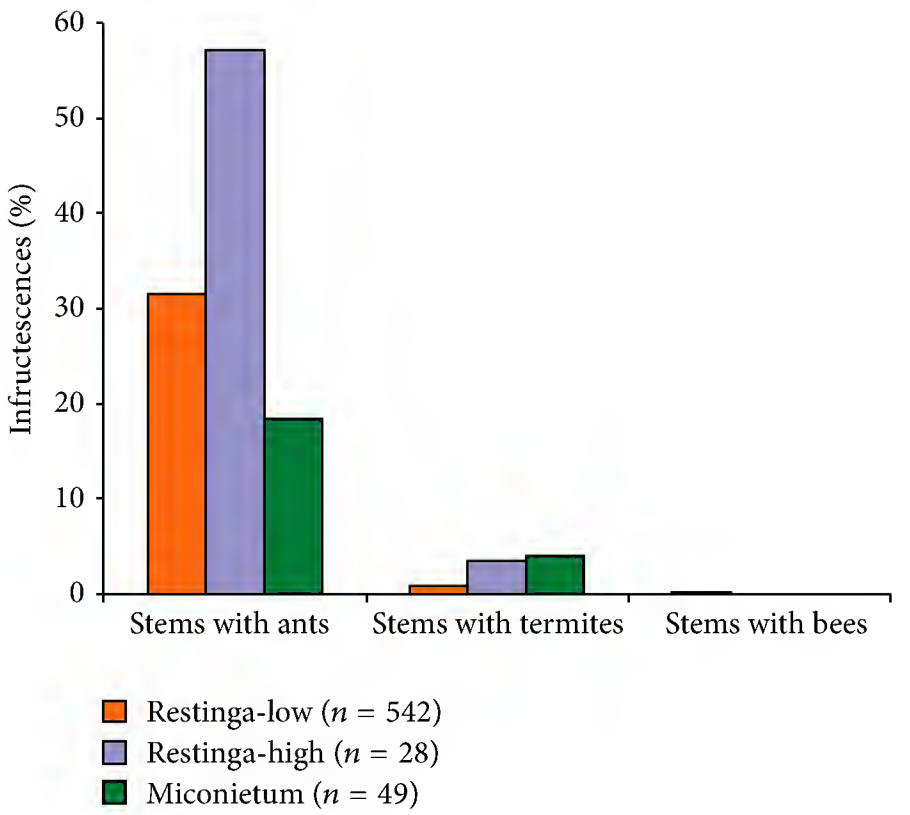

FIGURE 1: Percentages of infructescences of *Vriesea friburgensis* occupied by ant, termite, or bee nests in different habitat types (whole dataset, $n = 619$) on Santa Catarina Island, southern Brazil.

Under bracts, ant nests were present on 4–11% of infructescences (Supplementary Table S7). The most frequent stem inhabitants were ants of the genera *Camponotus*, *Pseudomyrmex*, and *Solenopsis* (Tables 1 and 2). During regular collections, we found nests of 14 ant species, at least two termite species (Termitidae: Nasutitermitinae: *Cortaritermes* sp. and *Velocitermes* sp.; habitat types miconietum and restinga-low; all nests occupied stem and rosette), and one bee species (Tables 1 and 2). Ant species composition differed among the habitat types (Table 1). We encountered eight cases of two social insect species occupying different sections of the same infructescence stem (Supplementary Table S8). Occasional findings outside of the regular dataset comprised nests of further ant species; brood of a megachilid bee, a castniid moth, and syrphid flies; several coleopterans, pseudoscorpions, collembolans, and spiders (Supplementary Table S9).

Out of 488 infructescence stems collected at restinga-low sites, 402 (82%) were hollow, and of these, 161 (40%) were occupied by ants, termites, or bees (Table 2). Length of cavities in infructescences was 92 ± 22 cm (mean ± SD, $n = 5$), and inner diameter was 1.9 ± 1.3 mm at apex and 4.6 ± 0.9 mm at base, yielding an estimated volume of 37 ± 30 cm^3 (assuming a truncated-cone shape).

3.2. Inflorescence Visitation by Ants. We found ants foraging on 22 of 33 (67%) flowering inflorescences. Furthermore, at 36–70% (median 55%, $n = 5$) of the weekly examined flowering inflorescences ($40 \leq n \leq 79$), ants were observed inside flowers. In contrast, ants were present at one of 26 (4%) inflorescences with buds only. Nine ant species/morphospecies were identified; most species and visitor records belonged to the genera *Camponotus* and *Pseudomyrmex* (Table 1).

Table 1: Absolute frequencies of occurrence of ant species recorded at 33 flowering inflorescences as well as of ant nests in infructescence stems (subset of 131 infructescences) of *Vriesea friburgensis* in three habitat types on Santa Catarina Island, southern Brazil. Differences between sum of ant records and number of occupied infructescences are due to occupations of stems with two ant species.

Ant species	At inflorescences (restinga-low)	Nests in infructescences		
		Restinga-low	Restinga-high	Miconietum
Acromyrmex rugosus (Smith 1858)	1			
Camponotus arboreus (F. Smith 1858)		1		
Camponotus bonariensis Mayr 1868		4		
Camponotus novogranadensis Mayr 1870	11	1		
Camponotus rufipes (Fabricius 1775)	2			
Camponotus sexguttatus (Fabricius 1793)	4	1		
Camponotus trapezoideus Mayr 1870			1	2
Camponotus sp. 13	1			
Camponotus sp. 14		1		
Cephalotes minutus (Fabricius 1804)				1
Crematogaster curvispinosa Mayr 1862			3	
Crematogaster limata F. Smith 1858			4	3
Nesomyrmex spininodis (Mayr 1887)				2
Procryptocerus convergens (Mayr 1887)				2
Pseudomyrmex gracilis (Fabricius 1804)	6	6		1
Pseudomyrmex phyllophilus (F. Smith 1858)	6	4		
Pseudomyrmex sp. PSW05[a]	1			
Solenopsis sp. 2	2	1	10[b]	1
Sum of ant records	34	19	18	11
Number of occupied stems	—	18	17	9
Number of examined stems	33	54	28	49

[a]A species of the *P. flavidulus* species complex which "might actually correspond to *P. flavidulus* itself" (Philip Ward, pers. comm.).

[b]Once three stems very close to each other were occupied by this species, so it was presumably the same colony, resulting in eight independent findings in restinga-high. Therefore, the value "8" was used for computation of similarity and diversity indices.

3.3. Species Richness and Diversity. The species accumulation curve of the restinga-high habitat reached a plateau (Supplementary Figure S5) and was significantly lower than the curve of miconietum, indicated by non-overlapping 95% confidence limits at the end of the shorter curve (Figure 2(a)). Moreover, the confidence limits of the restinga-high curve almost fell below those of the restinga-low curve. The other three curves lay near to each other without significant differences and without stabilizing. The Chao2 species richness estimator predicted highest (and even rising) richness for restinga-low and (according to the 95% confidence limits) significantly lowest for restinga-high (Figure 2(b)). The Simpson diversity index showed the same trend as the species accumulation curves (Figure 2(c)): highest diversity in miconietum and lowest in restinga-high. Finally, evenness was highest for miconietum (Figure 2(d)).

Comparing the ant communities that nested in infructescences, the Chao-Jaccard similarity index was highest for the habitat type pair restinga-high/miconietum (0.58), followed by restinga-low/miconietum (0.19) and restinga-low/restinga-high (0.05) (the latter significantly different from the first, according to 95% CIs), that is, beta diversity ascended in that order (Figure 3). Similarity between the inflorescence and infructescence samples in restinga-low was highest overall and significantly higher than between infructescence occupation in restinga-low and the other two habitats (Figure 3).

4. Discussion

4.1. Species Accounts. Altogether, we recorded 22 ant species (three subfamilies, nine genera) and at least 12 other arthropod species associated with inflorescences and infructescences of *V. friburgensis*. Even these high numbers must still be regarded as underestimations because species richness did not reach saturation in any habitat. Considering this inventory incompleteness and our sampling bias (focusing the search on social insects), there must be a lot more to discover in terms of animal associations with *V. friburgensis*. This is especially true if the view is extended from the inflorescence to the whole plant. The rosettes, which were not systematically examined in this study, might harbor a high diversity of macroinvertebrates as indicated by Zanin and

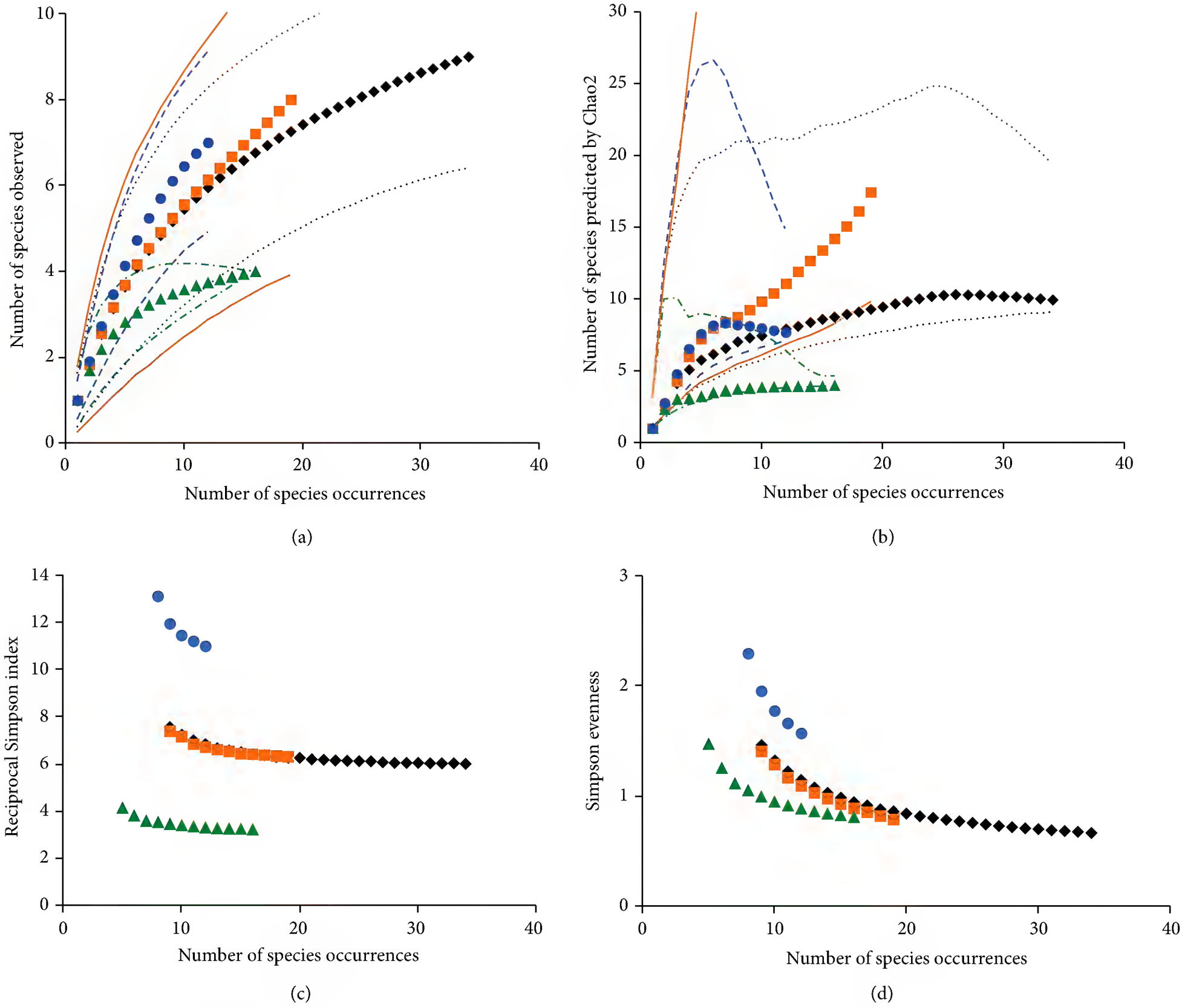

FIGURE 2: Diversity of ants inhabiting infructescences or visiting inflorescences of *Vriesea friburgensis*, according to habitat type on Santa Catarina Island, southern Brazil. Plotted against number of species occurrences as measure of sampling effort are (a) species accumulation curves and their 95% confidence limits; (b) Chao2 species richness estimator including 95% confidence limits; (c) rarefaction curves of Simpson diversity index; and (d) Simpson evenness (Simpson diversity divided by the number of species observed). Diagrams with the same data but with logarithmically scaled x-axes are included in the Supplementary Material (Supplementary Figure S5).

Tusset [15] for *V. friburgensis* and as reported for the related bromeliad species *V. inflata* (Wawra 1883) [21].

At least two ant species were for the first time reported for Santa Catarina Island (*Procryptocerus convergens*) or even Santa Catarina state (*Cephalotes minutus*) since they do not appear in previous inventories [7, 13, 22–30].

The termites we found living in the bromeliads probably belong to two undescribed species (E. Marques Cancello, pers. comm.). Since their nests were mostly located both in the rosettes and infructescence stems, their relation to the bromeliads might be similar to the association between the termite *Cortaritermes silvestrii* (Holmgren 1910) and the bromeliad *Dyckia maritima* Baker where the plants appear to grow on termite nests because of beneficial nutrition [31]. But this assumption needs further investigation to be confirmed.

4.2. Arthropods Living in Infructescences. We consider infructescences of *V. friburgensis* to be attractive nest sites for certain groups of arthropods (e.g., small ant colonies and small, serially nesting bees) because they

Table 2: Nests of ants and other insects in 488 infructescences (161 occupied) of *Vriesea friburgensis* in Joaquina (habitat type restinga-low) on Santa Catarina Island, southern Brazil. *n*: number of findings (total of 164 due to three cases with two nests in the same stem); % (occ.): percent fraction of the number of occupied stems; % (total): percent fraction of the number of examined infructescences.

Taxon	*n*	% (occ.)	% (total)
Apidae			
Ceratina (*Rhyssoceratina*) sp. (Xylocopinae)[a]	1	0.6	0.2
Formicidae			
Brachymyrmex	3	1.9	0.6
Pseudomyrmex	63	39.1	12.9
Pseudomyrmex gracilis	57	35.4	11.7
Pseudomyrmex sp. PSW05	6	3.7	1.2
Camponotus	68	42.2	13.9
Myrmelachista	1	0.6	0.2
Solenopsis	24[b]	14.9	4.1
Small yellow formicine or dolichoderine ants	1	0.6	0.2
Isoptera[c]	3	1.9	0.6

[a]Three females reared from brood cells.
[b]Once, five stems very close to each other were occupied by this species, so it was presumably the same colony, resulting in 20 independent findings.
[c]Probably *Cortaritermes* sp. according to another termite sample from the same location.

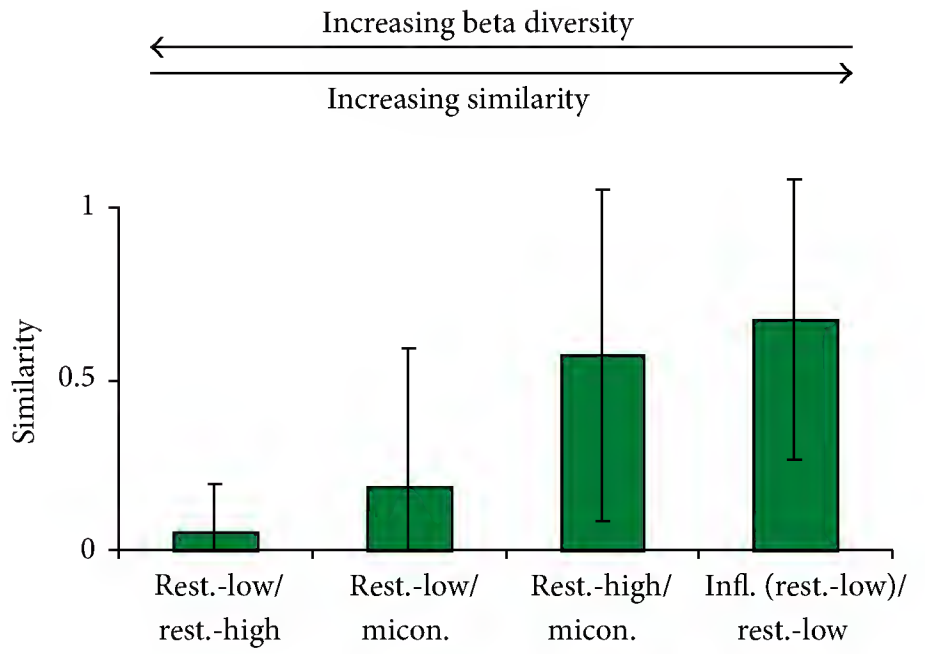

Figure 3: Chao-Jaccard similarity index for comparisons of infructescence occupation of *Vriesea friburgensis* by ants among habitats and of infructescence- with inflorescence-associated ants in restinga-low. Whiskers represent 95% confidence intervals; for convenience their lower bounds were cut when crossing the *x*-axis. Calculations were based on the data given in Table 1.

(1) provide a long, narrow space which can be used and defended more efficiently than a compact space of the same volume;

(2) dry up and frequently remain stable for more than a year, a feature which is important for bees and wasps with annual or bivoltine life cycles [9, 32];

(3) are mostly connected to a water reservoir in the leaf rosette, building kind of an oasis especially in well drained sandy habitats such as restingas;

(4) grow near to food sources, such as future inflorescences emerging from adjacent rosettes of the same bromeliad clone, providing floral nectar and flower visitors.

Not only can arthropods profit from nest space provided by *V. friburgensis* but also the plant may gain benefits. As known from former studies, ants frequently protect the plants they live in from herbivores (e.g. [33–36]). Whether *V. friburgensis* actually is protected from its inflorescence herbivores (e.g. *Eurytoma* sp. [9], *Strymon serapio* [10]) is unclear since many of them might already be active before the first flowers open; that is, when ants are not present yet. This might be assessed with exclusion experiments (e.g., [37]).

In most of the habitats studied, *Vriesea* infructescences appear to support a similar alpha diversity of ants. Whereas all diversity measures employed accord that restinga-high had lowest alpha diversity, with no more than four species predicted, species accumulation curves do not allow distinguishing among the other habitat types because they were too close to each other and did not stabilize. Moreover, the habitat ranking derived from the species richness estimator contradicts that indicated by the Simpson diversity index. Since the latter is influenced by species abundance distributions [18] we assume that restinga-low contains more species but with a rather uneven distribution in contrast to miconietum. This is indeed confirmed by estimated evenness.

The restinga-high habitat, in spite of its relatively low species richness, complements the ant species composition of the other habitats, especially low-vegetation restinga, demonstrated by the low similarity index value. Hence, occurring in such different habitats, *V. friburgensis* also supports a high beta diversity of ants.

We do not expect ants to be exclusively dependent on the infructescence stems as nest sites because similar cavities can also be found in other plants. For example, we discovered nests of *Pseudomyrmex gracilis* and *Ps.* sp. PSW05 in twigs of *Epidendrum fulgens* Brongn. 1834 (Orchidaceae) as well as *Nesomyrmex spininodis* in twigs of shrubs (V. S. Schmid, unpubl. data), and Cereto et al. [38] collected, at another restinga-low site on Santa Catarina Island, nests of eight ant species from postreproductive plants of *Actinocephalus polyanthus* (Bong.) Sano (Eriocaulaceae), a plant with a habitus similar to bromeliads and occurring sympatrically with *Vriesea* at our restinga-low study sites.

Cereto et al. [38] reported that 79.1% of *A. polyanthus* plants contained ant nests with up to four species living in the same plant. On the one hand, comparison with our study is difficult because sample sizes greatly differ, no accumulation curves were provided by Cereto et al. [38], and they examined whole plants while we only systematically examined the infructescence stems. It is well known that bromeliad rosettes are frequently used by ants as nest sites [6, 7, 13], so the percentage of *V. friburgensis* plants containing ant nests will most likely be higher than the occupation ratio of infructescence stems. On the other hand, both

Cereto et al. [38] and we report the occurrence of several ant species sharing the same plant, indicating that there might be high competition for nest sites in the restingas (see also Livingston and Philpott [39] arguing for generally high competition among ants). If this is true, it seems strange that a high proportion (61%) of hollow *V. friburgensis* infructescence stems was found unoccupied. The causes of this phenomenon remain to be studied in more detail, taking into account aspects such as dynamics of cavity development in plants and of ant colony movements, that is, turnover of site occupation.

4.3. Ants Visiting Inflorescences. Simpson diversity of ants inhabiting infructescence stems in restinga-low was similar to that of those visiting inflorescences in the same habitat type, although the estimated species richness differed significantly. Similarity in alpha diversity goes in line with the compositional similarity between these samples being higher than among the habitats, thus supporting our hypothesis that there is a great overlap between ant species that live in the bromeliads and those that visit their flowers.

However, individuals of some ant species visited the inflorescences, whereas the same species were not found living in infructescences. This may be due to nesting preferences; for example, *Camponotus rufipes* has large workers and colonies that construct nest mounds using plant material, sometimes within groups of *Vriesea* rosettes but apparently not extending into infructescence stems emerging from those rosettes; and *Acromyrmex* species generally nest in the soil [40].

Three *Camponotus* species nested in infructescence stems but were not observed on inflorescences. It might turn out interesting to find out the causes for this pattern, that is, whether it was mere chance owing to low sample size or whether these ants systematically avoid inflorescences, be it due to interspecific competition or because of their foraging habits.

We found five ant genera at inflorescences of *V. friburgensis*. However, within flowers, almost exclusively *Camponotus* ants were present (mainly *Ca. novogranadensis* and *Ca. rufipes*, probably also *Ca. sexguttatus*; V. S. Schmid, pers. obs.). They do not entirely monopolize the inflorescences since we mostly found unoccupied flowers near the occupied ones on the same inflorescences. Occasional behavioral observations indicate that *Camponotus* workers visit flowers to take up floral nectar, sometimes apparently guarded by a conspecific worker (Supplementary Figure S6). They might additionally hunt flower mites which we found along our examinations within flowers of 29 out of 32 (91%) inflorescences in Joaquina and also recorded them in miconietum (V. S. Schmid, unpubl. data), as similarly reported by Schmid et al. [13] for the bromeliad species *Aechmea lindenii* (E. Morren) Baker and *Ae. nudicaulis* (L.) Grisebach. We expect the mites in *V. friburgensis* to belong to the same species (*Proctolaelaps* sp. and *Tropicoseius* sp.) as in *Ae. lindenii* because they are phoretically transported by hummingbirds (see Video S6 in [13]) which occur on the whole island and visit flowers of species of *Aechmea* [3–5, 41] and *Vriesea* [4, 12].

As for infructescence occupation, we regard it as unlikely that any of the ant species reported here is specifically associated with inflorescences of *V. friburgensis*. Its flowers are accessible only during a limited period throughout the year and are not completely monopolized by one or a few ant species; thus ants do not completely depend on the floral resources.

The presence of ants on plants is frequently accompanied by a mutually beneficial association where the ants are attracted by food and/or shelter and in turn provide protection to the plant by deterring herbivores and/or cutting other vegetation that competes with the host plant for resources (e.g., [33–36]). In bromeliads, such a mutualism was reported for *Dyckia floribunda* where exclusion of ants yielded a significant decrease in total seed production per plant [37]. Unlike *D. floribunda*, *V. friburgensis* does not produce extrafloral nectar on its inflorescences (V. S. Schmid, unpubl. data: six plastic-bagged inflorescences inaccessible to animals did not show signs of secreted fluids). The ants are apparently attracted mainly by the nectar contained inside the flowers. They might have both positive and negative effects on the plants' reproductive success by interfering with herbivores and/or pollinators, respectively. Hence, whether *V. friburgensis* benefits from the presence of the ants cannot be judged without appropriate manipulative experiments.

5. Conclusion

Even though there are probably no specific associations with *V. friburgensis*, this bromeliad species does support a high level of alpha and beta diversity of arthropods, mainly ants. Regarding the high potential for competition for nest sites among ant species [39], *V. friburgensis* likely plays an important role for the species communities of the Atlantic Forest region, confirming former studies which stressed the ecological significance of bromeliads (e.g., [10, 13, 42, 43]; see also [2] and references therein). Beyond the scope of our study, there are certainly other bromeliad species (e.g., *Dyckia* spp.; *Hohenbergia* spp.; other *Vriesea* spp.) whose infructescences are worth a closer examination with respect to inhabiting arthropods. Concluding, we recommend that bromeliads should be taken into special consideration for biodiversity conservation efforts.

Conflict of Interests

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

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Research Article

Volatile Organic Compounds from the Clone *Populus x canadensis* "Conti" Associated with *Megaplatypus mutatus* Attack

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Megaplatypus mutatus (Chapuis) (Coleoptera, Platypodidae) is an ambrosia beetle native to South America. It builds internal galleries that weaken the tree trunks, causing them severe stem breakage and mortality in commercial poplar plantations. The host selection by male *M. mutatus* has previously been correlated with the increasing diameter. This work explores the possibility that differential susceptibility of individual plants to *M. mutatus* could be associated with volatiles emitted. The comparison of the VOCs profiles of attacked and nonattacked *P. x canadensis* "Conti" 12 during *M. mutatus* flying season showed both qualitative and quantitative differences. The attacked plants, but not the nonattacked ones, showed the following compounds: a long chain aldehyde, α-ylangene, δ-cadinene, α-gurjunene, and β-cubebene; on the other side, β-sesquiphellandrene and β-chamigrene were detected only in nonattacked plants. α-Copaene is a common component of all the samples analyzed, but its proportion is increased in attacked individuals. Behavioral bioassays showed that males but not females *M. mutatus* are attracted to α-copaene. The relative increase of α-copaene in attacked individuals and the positive behavioral answer of males to it suggest that this compound could play a role in the orientation of the pioneer male towards the most suitable host.

1. Introduction

Ambrosia beetles are an important insect group in forest ecosystems affecting weakened or felled trees. *Megaplatypus mutatus* (Syn. *Platypus mutatus*) (Chapuis) (Coleoptera, Platypodidae) is an ambrosia beetle native to South America. Unlike most ambrosia beetles, it attacks only living trees, penetrating the xylem of its host by boring long tunnels. The attack is initiated by pioneer males selecting a host tree to build a short nuptial gallery, from which they attract females using a sexual pheromone [1]. Following copulation, they extend their gallery in order to lodge their new brood. These galleries weaken the tree trunks, causing severe stem-breakage and mortality in commercial poplar plantations of *Populus deltoides* [2–4]. Additionally, the dark tunnels caused by the Ambrosia mycelium of the associated symbiotic fungi seriously affect the quality of the wood.

The prevalence of attacks by *M. mutatus* has been correlated with the tree diameter. Etiennot et al. [5] found that 86% of the attacked trees in a plantation had a diameter breast height (DBH) >20 cm. Also, other authors found a preference of *M. mutatus* for bigger diameters [6–9], probably because there is more room available to develop their brood [1].

Concerning the susceptibility associated with the clone, although it is well known that some clones are less susceptible than others, this differential susceptibility is more likely to be associated with the average DBH of the particular clone characteristic for its growing rate than to the clone itself [6–9]. Also, there is a strong association between the site quality and the prevalence of attacks [10]. Again, this phenomenon can be correlated with the productivity of the plantation.

With the aim of implementing an environmentally friendly management programme, a large amount of work has been done with traps baited with sexual pheromone that

attract females [11–13]. However, the existence of chemical cues involved in the host selection by the male has not been explored and it gains interest in the search of synthetic attractants to be incorporated in baited traps. In this work we explore the possibility that differential susceptibility of individual plants at *M. mutatus* could be associated with VOC emitted, so we collected and analyzed VOC emitted by wood bark of the clone *P. x canadensis* "Conti" 12 attacked and nonattacked during *M. mutatus* flying season.

2. Materials and Methods

2.1. Plant Material. *Populus x canadensis* Mönch (Syn. *P. x euramericana* (Dode) Guiner) plants were selected from 10-year-old commercial poplar plantations (*Populus x euramericana* cv. "Conti 12").

The plantation had a density of 1,111 trees/ha (square of plantation 3 m × 3 m), average DBH 23,2 cm and is located at Alberti, Buenos Aires Province, Argentina (35°10′S, 60°17′W, 68 m.a.s.l.). All the selected individuals had the same age, site, clone, and history of plantation.

The selection methodology was the following: we randomly selected an attacked plant and a nearby nonattacked one with a similar diameter. We also tried to select attacked plants close to nonattacked one and vice versa. Trees were considered attacked if they had a visible pioneer calling male, characterized by the presence of a crown-like arrangement surrounding the entrance to the gallery (Figure 1). Four replicates of attacked and nonattacked trees were analyzed. Samples were collected during the flying season of *M. mutatus* (November).

2.2. Volatile Organic Compounds Emitted by Populus x euramericana cv. "Conti 12". Using a cork borer we extracted a wood bark cylinder vicinal (1.5 cm × 1 cm) to the *M. mutatus* entrance hole and placed it in a 20 mL vial standard clear glass (Scientific Specialties Service, Inc., Baltimore, MD, USA and Reno, NV, USA) with a teflon-coated cap (teflon septum with glass reinforced polypropylene resin open cap) adequately refrigerated. All the samples were collected between 10 and 12 a.m.

Once in the lab, the volatiles from the vial headspace were collected at 29 ± 2°C for 30 minutes using a solid phase microextraction fiber (SPME) covered with a 100 μm PDMS (Supelco Bellefonte, PA, USA) nonpolar phase. This coating is of general use to adsorb low molecular weight compounds. Samples were immediately analysed by GC-MS. GC-MS analyses were performed with a Shimadzu QP-5050A spectrometer in the electron impact mode, equipped with a polar fused CP wax 52CB column (30 m × 0.32 mm ID × 0.25 μm film thickness). Samples were injected in the splitless mode. Volatiles from the SPME fibres were desorbed in the injector port at 250°C during 1.5 min. The GC column was kept at 50°C for 5 min after which the temperature was programmed to increase 10°C/min up to 220°C, where it was maintained for 5 min. The carrier gas was helium with a head pressure of 30 kPa. The MS detector was set on at 70 eV.

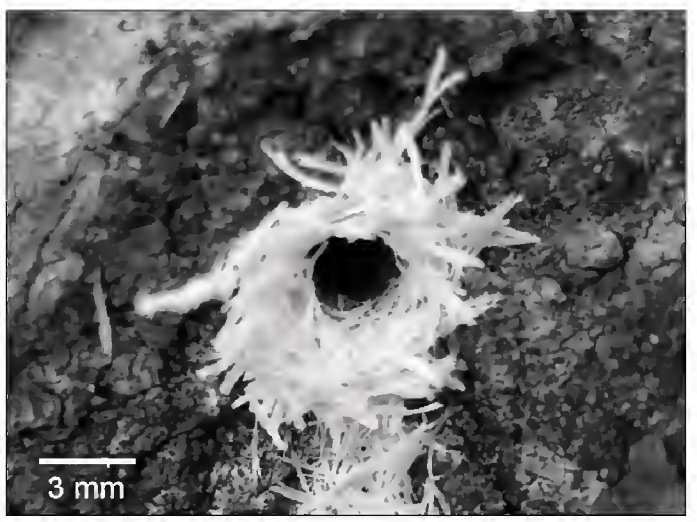

FIGURE 1: Crown-like arrangement in *P. canadensis* surrounding the entrance to the nuptial gallery built with the particles of boring dust (frass) produced by the male *M. mutatus* from where volatiles are emitted to attract individuals of the opposite sex.

The identities of compounds observed were assigned by comparison with spectral data of commercial libraries NIST and Wiley (tentative identifications) or with the authentic compound in the case of α-copaene.

2.3. Insects. The insects were collected shortly after their emergence (maximum 3 hours) from infested *Populus* sp. and *Quercus palustris* (Münchh) located at our institute plantation (34°33′ south, 58°30′ west). Emergence traps specifically designed for this beetle were used to avoid antagonistic interactions between emerged insects [14].

2.4. Behavioral Bioassays. Walking behavior of female *M. mutatus* was evaluated in an experimental arena with a video tracking technique [15] adapted for *M. mutatus* [16]. The floor of the test arena was covered with a round piece of Whatman No. 1 filter paper (125 mm diameter, Whatman Ltd., Maidstone, UK), and a glass cover (20 × 20 mm) was placed in the center of the paper. Next, the filter paper and glass cover were both covered with a rectangular piece of wire mesh (100 × 100 mm, 1 mm mesh size). A colorless glass ring (100 mm diameter, 50 mm high) was used to confine the insects. A new glass cover and filter paper were used in each replicate.

A closed circuit video camera providing black and white images (VC 1910, Sanyo Electrical Co., Tokyo, Japan) was suspended 22 cm over the center of the test arena. A circular fluorescent tube (22 W, OSRAM, Buenos Aires, Argentina) was placed 64 cm above the video camera.

An image analyzer (Videomex V, Columbus, OH, USA) received input from the video camera, converting the analog signal into digital data. The resolution was 256 × 192 pixels and the acquisition and processing speed was 30 frames/sec. The presence of insects in the arena was determined by visual contrast between the individuals (white) and the arena background (dark) and scored as the number of "*ON*" pixels. The area occupied by the insects was recorded by using the Multiple Zone Motion Monitor for Videomex software.

The arena image was divided into a central square (4 cm^2, 5% of the total area) and a circular outer area. The center of the glass cover was located in the center of the virtual central square. A male *M. mutatus* was placed on the wire mesh and allowed to acclimatize for 5 min before starting the bioassay. During this time, the insect moved all around the arena. Insect movement was recorded for 60 min. During the first 30 min, the glass cover was clean. Then, 1.5 μL of α-copaene was placed on the cover. Temperature varied between 25 and 30°C. The first 30 min of each test was the control, and the remaining 30 min was the experimental treatment. Thus, the occupation level of the central circle during the first 30 min (control) was compared to the occupation level during second 30 min (following the introduction of the test substance). The experiment was replicated 10 times with independent males and females.

We used the central area of occupation (CAO) parameter, previously defined as the total number of "*ON*" pixels in the central circle (where the test compound is placed) during a replicate [15, 16], to quantify insect behavior. A mean CAO value was obtained for each treatment and compared to its respective control.

2.5. Chemical. (−)-α-Copaene (Technical grade > 90%, GC sum of enantiomers) was purchased from Fluka (Milwaukee, USA).

2.6. Statistical Analysis . Data from the behavioral assay were analyzed by Kruskal-Wallis Test (nonparametric ANOVA) using STATISTICA software. A mean CAO values were obtained for α-copaene and compared to its respective control. The accepted level of significance was P value < 0.01, meaning highly different from control group (Kruskal-Wallis Test).

The values of relative concentration of the compounds for each sample were transformed (log) and analyzed using one-way analysis of variance (ANOVA), and means were compared a posteriori by Tukey HSD mean multiple comparison test using STATGRAPHICS Plus Software. A value of $P < 0.01$ was considered for a significant highly difference and $P < 0.05$ for a significant difference.

3. Results

All the specimens of *Populus x canadensis* clone "Conti 12" whose volatiles where analyzed have the same age, site, clone, diameter, and history of plantation. The attacked ones had a DBH 25.5 ± 1.63 cm and the nonattacked ones 20.7 ± 1.96 cm. This means that among a similar diametrical class, the insect prefers the larger diameters (P value < 0.05).

3.1. Volatile Organic Compounds Emitted by Nonattacked Populus x canadensis Clone "Conti 12". The volatile blend emitted by the wood and bark sample of the *P. x canadensis* "Conti 12" nonattacked by *M. mutatus* was dominated by β-selinene (36.9 ± 2.6%), followed by α-selinene (27 ± 3.0%), β-chamigrene (7.1 ± 2.6%), a long chain aldehyde with $Rt_{21.82}$ (6.3 ± 1.8%), β-elemene (5.0 ± 2.6%), salicylic aldehyde (3.5 ± 2.0%), and α-copaene (1.8 ± 0.2%) (Figure 2(a)).

3.2. Volatile Organic Compounds Emitted by Attacked Populus x canadensis Clone "Conti 12". Figure 2(b) shows the typical GC trace of the volatiles emitted by the wood and bark sample of the *P. x canadensis* "Conti" 12 attacked by *M. mutatus*. In this case α-copaene was the major component (34.4 ± 23.9%), followed by a long chain aldehyde of Rt 20.57 (30.7 ± 15.8%), β-selinene (9.1 ± 3.8%), a long chain aldehyde of Rt 21.82 (8.6 ± 4.7%), α-selinene (4.4 ± 3.0%), β-cubenene (2.2 ± 0.6%), salicylic aldehyde (2.1 ± 1.8%), α-gurjunene (1.8 ± 0.5%), β-elemene (1.7 ± 0.9%), α-ylangene (1.0 ± 0.6%), and δ-cadinene (0.9 ± 0.4%).

3.3. Behavioral Response to α-Copaene. The occupation level of the central circle during the first 30 min (control) did not reveal a significant behavioral response when compared with their second 30 min (following the introduction of the test substance) (P value: 0.001).

Results were analyzed based on the central area of occupation (CAO) parameter. Significant occupation of the central area can be interpreted as an effective attraction to the source followed by an arrestment in the area [17].

CAO values of female *M. mutatus* exposed to α-copaene did not reveal a significant behavioral response (P value: 0.62) when compared with their respective controls (Figure 3). Thus, females were not attracted to the stimulus source.

CAO values of male *M. mutatus* exposed to α-copaene revealed a significant behavioral response (P value: 0.0042) (Figure 4) when compared with their respective controls. Thus, males were attracted to the stimulus source.

4. Discussion

The comparison of the volatile profiles of attacked and nonattacked trees showed both qualitative and quantitative differences(Figure 5). The attacked plants, but not the nonattacked ones, showed the following compounds: a long chain aldehyde of $Rt_{20.57}$, α-ylangene, δ-cadinene, α-gurjunene, and β-cubebene; on the other side, β-sesquiphellandrene and β-chamigrene were detected in nonattacked plants but not in attacked ones.

A quantitative analyses showed that α-copaene is present in 1-2% in nonattacked plants but in 34, 4% in attacked ones (P value < 0.05).

Also, the long chain aldehyde of $Rt_{21.80}$ shows the same pattern: it varies from 6.3% in nonattacked plants to 30.7% in the attacked ones (significant difference, P value < 0.05). Instead, α-selinene, β-selinene, and β-elemene decrease their relative concentrations in attacked trees with respect to nonattacked ones (P value < 0.01, P value < 0.01, and P value > 0.05, resp.).

Overall, we can conclude that although α-copaene is a common confirmed component of all the samples analyzed, its proportion is increased in attacked individuals and males

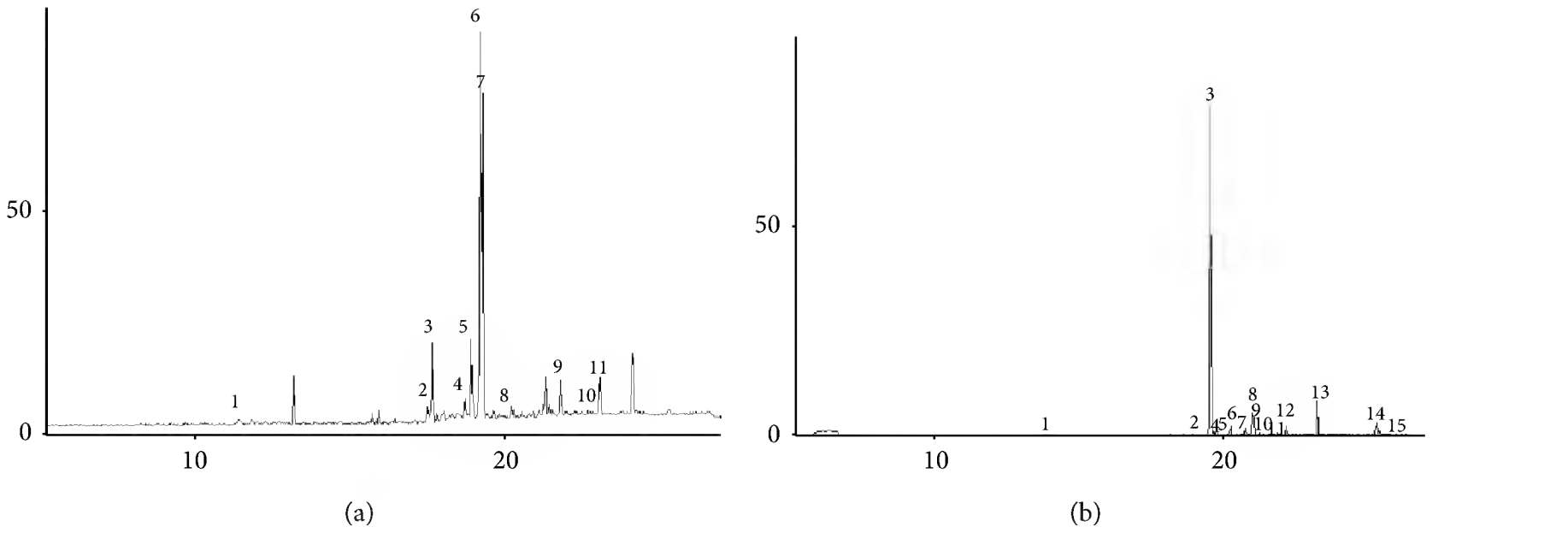

FIGURE 2: (a) Typical GC trace of volatile organic compounds emitted by nonattacked *Populus x canadensis* clone "Conti 12" (1: Salicylic aldehyde**, 2: α-copaene***, 3: β-elemene**, 4: N.I., 5: β-chamigrene**, 6: β-selinene**, 7: α-selinene**, 8: N.I., 9: aldehyde of $Rt_{21.82}$**, 10: N.I., and 11: N.I.). **: Tentatively identified against GC-MS library, ***: identified against authentic standard, and N.I.: nonidentified. (b) Typical GC trace of volatile organic compounds emitted by attacked *Populus x canadensis* clone "Conti 12" (1: salicylic aldehyde**, 2: α-ylangene**, 3: α-copaene***, 4: β-elemene**, 5: N.I., 6: β-cubebene**, 7: α-gurjunene**, 8: β-selinene**, 9: α-selinene**, 10: δ-cadinene**, 11: N.I., 12: aldehyde of $Rt_{20.57}$**, 13: aldehyde of $Rt_{21.82}$**, 14: N.I., and 15: N.I.). **: Tentatively identified against library, ***: identified against authentic standard, and N.I.: nonidentified.

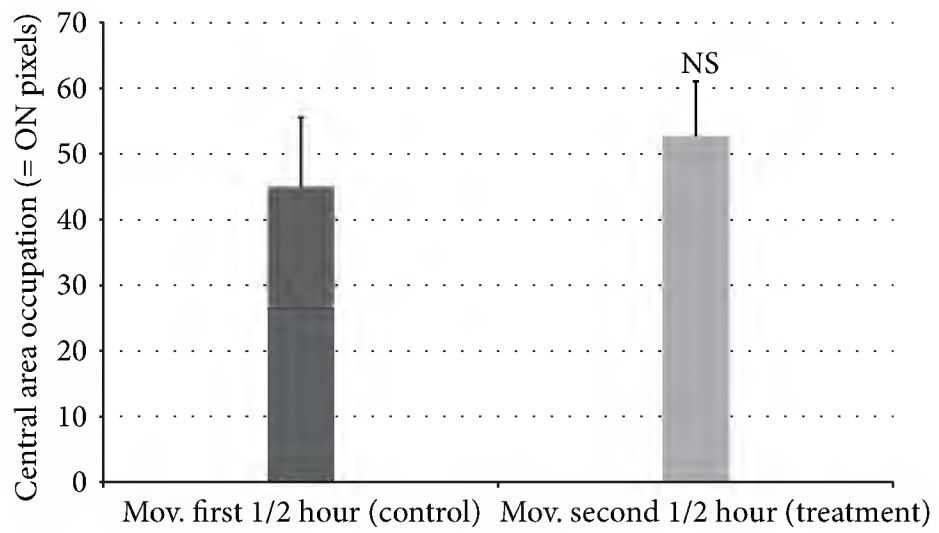

FIGURE 3: Response of female *Megaplatypus mutatus* measured as the central area occupation (=*on* pixels) for α-copaene compared to its respective control. Each bar represents the mean of 10 independent replicates ± SE. NS: not significant differences between treatment and control group (Kruskal-Wallis Test, $P > 0.01$).

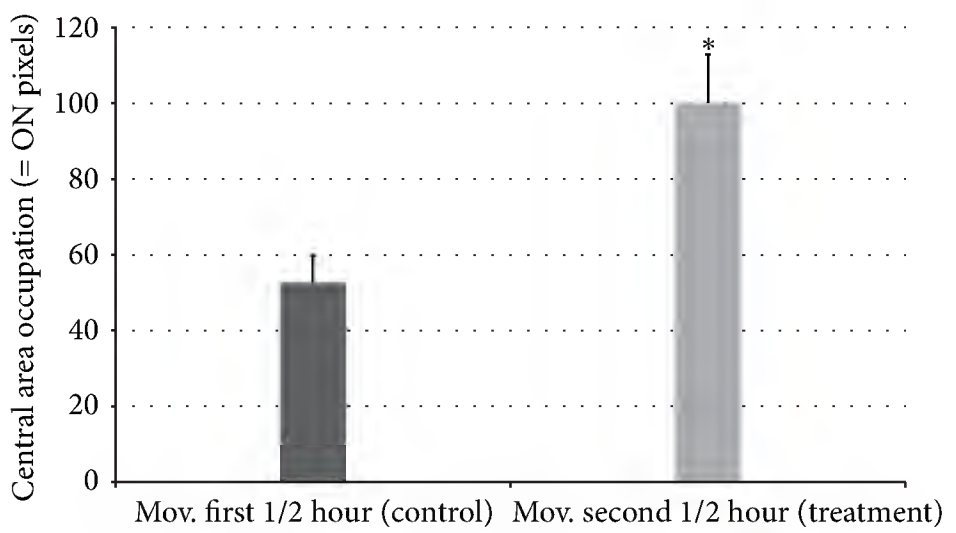

FIGURE 4: Response of male *Megaplatypus mutatus* measured as the central area occupation (=*on* pixels) for α-copaene compared to its respective control. Each bar represents the mean of 10 independent replicates ± SE. *: significant differences between treatment and control group (Kruskal-Wallis Test, $P < 0.01$).

M. mutatus are attracted to it at short range but females are not.

The relative increase of α-copaene in attacked individuals and the positive behavioral answer of males to it suggest that this compound could play a role in the orientation of the pioneer male towards the most suitable host.

α-Copaene and its stereoisomer α-ylangene are active kairomones of *Archangelica officinalis* essential oil; however, their proportion goes from 0.5 to 1% and pure α-copaene is quite more active. The Angelica essential oil has been used in baited traps to catch fruit flies in Florida [18]. Also, extracts of *Litchi chinensis, Ficus retusa*, and *Ficus benjamina* were active for males of the same species being this response attributed to the presence of α-copaene [19].

Our result is interesting for our goal of finding natural attractants to be set up in baited traps in the field. Attraction of bark beetles to pheromone baited traps is increased by the addition of host volatiles as monoterpenes to pheromone baits [20, 21] and commercial lures based on the combination of synthetic attractants are available. In this sense, the introduction of α-copaene to pheromone baited traps could be a promising tool that optimizes adult trapping, leading to improve monitoring and control systems in infested plantations.

5. Conclusions

The volatile profiles of attacked and nonattacked trees showed both qualitative and quantitative differences.

α-Copaene is a common confirmed component of all the samples analyzed, but its proportion is increased in attacked individuals.

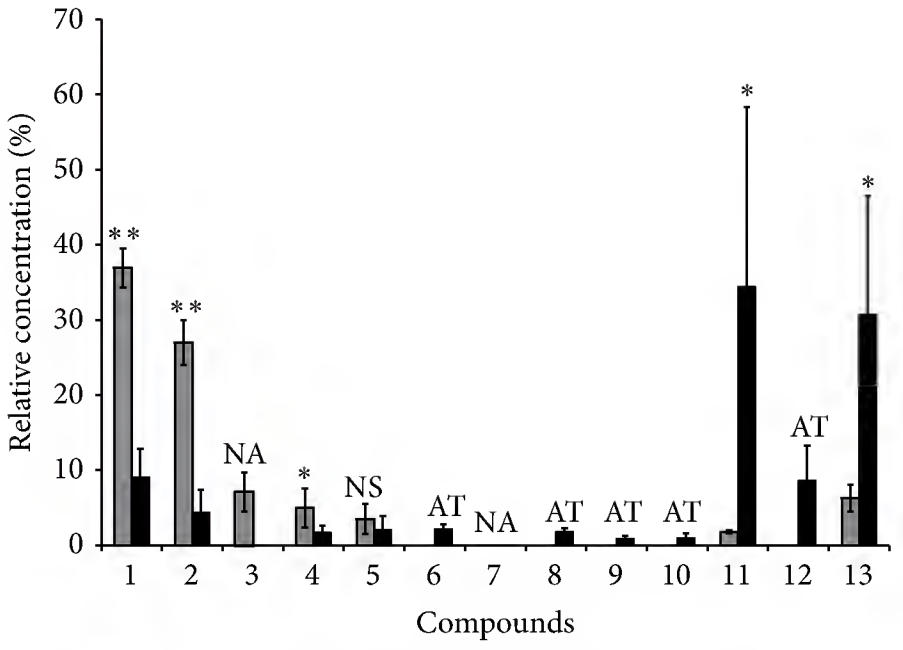

FIGURE 5: Volatile organic compounds emitted by attacked and nonattacked *Populus x canadensis* clone "Conti 12." The numbers represent the major compounds, 1: β-selinene, 2: α-selinene, 3: β-chamigrene, 4: β-elemene, 5: salicylic aldehyde, 6: β-cubebene, 7: β-sesquiphellandrene, 8: α-gurjunene, 9: δ-cadinene, 10: α-ylangene, 11: α-copaene, 12: aldehyde of $Rt_{20.57}$, and 13: aldehyde of $Rt_{21.82}$. The area normalization performed only on identified compounds and the values are the mean of four replicates ± SD. NS: not significant differences between relative concentration in attacked and non-attacked plants ($P > 0.05$). * and **: relative concentration in the attacked plants is significantly different ($P < 0.05$) or highly different ($P < 0.01$) respectively, from non-attacked plants (ANOVA-Tukey HSD mean multiple comparison test). AT: compounds only present in attacked plants and NA: only present in nonattacked plants.

In behavioral bioassays, males *M. mutatus* are attracted at short range to α-copaene, while females are not.

Introduction of α-copaene to pheromone baited traps could optimize adult trapping.

Conflict of Interests

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

Acknowledgments

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Research Article

Efficacy of Neem Oil on Cardamom Thrips, *Sciothrips cardamomi* Ramk., and Organoleptic Studies

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The neem tree contains promising pest control substances which are effective against many pests. Oil extracted from neem seeds was used against cardamom thrips, *Sciothrips cardamomi*, a severe and economic pest of cardamom. Neem oil formulations, namely, Tamil Nadu Agricultural univeristy neem oil (TNAU NO) (acetic acid & citric acid), were found effective against the pest with a overall damage reduction of 30% after 14 days of treatment. The percent damage reduction in capsules over control after three consecutive sprays of TNAU NO(C) 2% and TNAU NO(A) 2% was 78.3 and 75.2 percent, respectively. The newly extracted and unformulated neem oil, though found inferior to the formulated one, still found to cause 50% and 70% reduction in damage caused by thrips at two and three rounds of sprays, making it useful in pest management. Organoleptic tests conducted on cardamom capsules sprayed with neem oil revealed no significant difference in taste, aroma, and overall acceptability of cow milk boiled with cardamom. Thus, TNAU NO (A and C) 2% was found effective against cardamom thrips with no adverse organoleptic properties and can be recommended.

1. Introduction

The backlash of synthetic pesticides because of the residual, resistance, and nontarget effects has led to the exploration of ecologically safe pest control alternatives in crop production. Among the different plant species with insecticidal properties, neem (*Azadirachta indica* A. Juss) is the well-studied and most commercially exploited one for pest management. Azadirachtin, a tetranortriterpenoid, was reported active over nearly 550 insect species [1]. Neem based insecticides especially those having azadirachtin are very much required for IPM programmes because they are selectively toxic, nonbioaccumulating, less persistent, and a natural source of insecticides [2]. Mode of action of neem on insect pests include direct effects on insect reproduction and secondary antifeedancy, and the physiological effects, measured as growth reduction, increased mortality and abnormal and delayed moults [3]. Neem seed kernel extract (NSKE), neem oil (NO) and neem cake (NC) are used in various field and horticultural crop pest managements. Neem oil cannot be used as such and has to be formulated to increase its efficacy and to decrease the potential phytotoxicity and to increase the storability. Neem oil per se is less systemic because it is insoluble in water. It should be formulated to make it systemic, to enhance its efficacy on sucking pests. To overcome these hurdles, better formulations are being developed [2]. Two neem oil formulations were made available by Tamil Nadu Agricultural University namely, TNAU NO(C) and TNAU NO(A) which are being tested for the efficacy on different insect pests. The neem product, TNAU NO(C) 30 mL/L, was reported effective against okra leaf hopper, *Amrasca devastans*, and reduced the population by 90% in one week period [4]. Both the formulations A and C at 3% were reported effective against sesame shoot webber and capsule borer, *Antigastra catalaunalis*, also [5]. TNAU NO is found effective against many other pests like *Liriomyza trifolii* on cotton [6], *Amrasca biguttula* and *Aphis gossypii* in okra [7], *Hypothenemus hampei* in coffee [8], *Pseudodendrothrips mori* in mulberry [9], and *Tetranychus urticae* in bhendi and brinjal [10].

Application of neem based formulations effectively checks insect pests of cardamom [11] and neem based IPM was also developed for cardamom borer and thrips [12].

Spraying neem oil 0.03% was found effective and caused 47% reduction in cardamom borer infestation [13]. Neem oil suspension at 0.5% sprayed on the lower surface of the leaf is very effective for the control of whitefly nymphs [11]. Margocide CK 0.1% effectively reduced root grubs in the field [14]. Neem cake 600 Kg acre^{-1} is also effective in controlling the grubs [15]. Moreover, neem cake was reported to significantly reduce the incidence of shoot fly of cardamom and also to enhance the production of side suckers [16]. The neem formulation under study is new, easy to make, cheap, and reported effective against many sucking pests of crops and thus needs to be evaluated against important pests of cardamom.

The reports of Jood et al. [17] stated that maize treated with neem oil, neem leaf, and kernel powder adversely affected the taste, aroma, and overall acceptability of Chapati rendering it unsuitable for consumption makes the necessity of organoleptic test especially for botanical pesticides. However, organoleptic tests conducted with broiler chicken fed with diets containing urea ammoniated neem seed kernel cake revealed no bitter taste in the cooked meat [18]. With these views, a study was carried out to find the efficacy of TNAU NO (A and C) along with unformulated neem oil and a commercial neem product (Vijay Neem) on cardamom thrips and organoleptic test on capsules collected from neem sprayed cardamom plants.

2. Materials and Methods

2.1. Efficacy Studies. Two field trials were laid out in cardamom plantations in Bodimettu, Bodi, during March to May, 2006, and Devarshola, Gudalur, Tamil Nadu, during September to November, 2006, to find out the efficacy of neem formulations on cardamom thrips. The trials were laid in randomized block design as per the treatments given in Tables 1 and 2 with three replications. The new neem formulations, TNAU NO (A and C), were made and standardized by Tamil Nadu Agricultural University, Coimbatore. The formulations are of 60% a.i and the first formulation contains acetic acid and thus is denoted as A and the other has citric acid and is denoted as C. The TNAU neem oil formulations A and C were tested at the rate of 2 and 3% each and compared with unformulated neem oil and a commercially available neem formulation (Vijay Neem).

Field trials were laid out in randomized block design (RBD) in the farmers' holdings in Bodimettu, Bodi and Devarshola, Gudalur, to test the efficacy of neem oil against thrips. Both the trials were conducted in a ruling variety of cardamom, namely, Njellani Green Gold, as per the treatments given in Tables 1 and 2 and replicated thrice. Spray treatment was given using backpacked knapsack sprayer with hollow cone nozzle at a rate of 750 L ha^{-1} (1500 cardamom clumps). Three sprays were given at 15 days interval and observations were made on the capsule damage. A control treatment was made by spraying only water.

The thrips incidence in cardamom was assessed on capsule basis and expressed as percent damage. Percent damage was assessed by counting total number of capsules per ten panicles in four clumps in a treatment and capsules showing scabs 3, 7 and 14 days after each application and also prior to the treatment. A clump consists of 5-6 cardamom plants planted/grown together, which covers an area of 0.8 to 1 m^2, demanding 0.5 L of spray fluid per clump. The percent damage thus recorded was subjected to statistical analysis adopting randomized block design using IRRISTAT version 3/93 after converting it to arcsine values. The mean values of treatments were then separated by Duncan's multiple range test (DMRT) after being transformed into arcsin values [19].

2.2. Organoleptic Test for Neem Sprayed Cardamom. Samples were collected 10 days after treatment from different treatments as given in Table 3 for TNAU neem oil sprayed plants from the field. Milk was boiled after putting these cardamom capsules separately for each treatment at 20 capsules per L of milk. To obtain unbiased scores each sample was coded. Organoleptic properties of milk for colour, aroma, taste, and overall acceptability were done by a panel of 10 judges. All are untrained panelists but well educated and most of them are agricultural professionals aged between 24 and 55 years. Using a well-structured questionnaire, the panelists independently assessed the samples for appearance (colour), taste, aroma/flavor, and overall acceptability employing 9.0 point hedonic scale [20] as given in Table 4.

3. Results

3.1. Field Trial I-Bodimettu. The mean damage by thrips prior to neem application ranged from 12.0 to 14.6 percent (Table 1). Three days after spraying, the capsule damage ranged between 11.5 and 13.3 percent in different treatments, while in the control it was 15.0 percent. The maximum mean reduction in capsule damage over check being 32.9 percent was recorded in TNAU NO(C) 3% followed by TNAU NO(A) 3% (31.5%) at the end of first spray. Ordinary neem oil 0.2% recorded the least reduction of damage over check (21.5%). Plots treated with TNAU NO(C) 3% and 2% registered a damage score of 10.8 and 9.6 percent and 10.7 and 9.6 percent 7 and 14 days after treatment, respectively, which were not significantly different from each other. The check, Vijay Neem at 2 mL L^{-1} recorded 11.8 7 days after treatment and 10.6 percent damage 14 days after treatment. Second spray was given fifteen days after the first spray when the damage ranged from 9.4 to 16.8 percent. At 7 days after treatment, TNAU NO(C) 3% and 2% recorded a damage of 7.8 to 8.1 percent which were on par with each other (Table 1). Though the reductions in capsule damage were low, the thrips population was reduced significantly in all the treatments after the sprays except untreated check. The same trend of efficacy was observed in the third spray also. TNAU NO(C) 3% recorded thrips damage to a level of 6.0, 4.9, and 3.8 percent 3, 7, and 14 days after treatment, respectively, and was found to be statistically superior to other treatments. TNAU NO(C) 3% was found superior in reducing the damage to a level of 80.1 percent at the end of three applications. The percent reduction over control after three sprays of TNAU NO(C) 2% and TNAU NO(A) 2% was 78.3 and 75.2 percent, respectively (Table 1).

Table 1: Effect of TNAU neem oil on thrips damage in cardamom-Bodimettu, Bodi (mean of three observations).

Treatments	PTC	First round of application					Second round of application					Third round of application				
		3 DAT	7 DAT	14 DAT	Mean	% Redn	3 DAT	7 DAT	14 DAT	Mean	% Redn	3 DAT	7 DAT	14 DAT	Mean	% Redn
TNAU neem oil (A) 2%	12.0	11.5^{a}	10.7^{a}	10.6^{b}	11.0	31.0	9.9^{b}	8.8^{b}	7.1^{b}	8.6	54.7	6.9^{ab}	6.1^{abc}	5.3^{bc}	6.1	75.2
TNAU neem oil (A) 3%	12.6	12.5^{bc}	10.4^{a}	9.4^{a}	10.9	31.5	9.0^{a}	8.9^{bc}	7.2^{b}	8.4	56.0	6.8^{ab}	6.0^{abc}	4.9^{abc}	5.9	76.0
TNAU neem oil (C) 2%	13.4	12.7^{bc}	10.7^{a}	9.6^{a}	11.0	30.5	9.0^{a}	8.1^{a}	6.9^{b}	8.0	57.9	6.4^{ab}	5.4^{ab}	4.3^{ab}	5.4	78.3
TNAU neem oil (C) 3%	12.0	11.5^{a}	10.8^{a}	9.6^{a}	10.7	32.9	9.0^{a}	7.8^{a}	6.4^{a}	7.7	59.5	6.0^{a}	4.9^{a}	3.8^{a}	4.9	80.1
Neem oil 2%	13.3	13.1^{c}	12.6^{c}	11.7^{c}	12.5	21.5	11.0^{c}	9.9^{d}	8.6^{d}	9.8	48.5	8.1^{b}	8.0^{d}	6.8^{d}	7.6	69.0
Neem oil 3%	12.4	12.0^{ab}	11.2^{ab}	10.7^{b}	12.0	24.5	10.1^{b}	9.4^{c}	8.0^{c}	9.2	51.9	7.7^{b}	6.9^{bcd}	5.7^{cd}	6.8	72.5
Vijay Neem 2 mL L^{-1}	14.0	13.3^{c}	11.8^{bc}	10.6^{b}	11.9	25.0	10.0^{b}	9.3^{bc}	8.1^{c}	9.2	51.9	7.9^{b}	7.0^{cd}	6.1^{cd}	7.0	71.5
Untreated check	14.4	15.0^{d}	15.8^{d}	16.8^{d}	15.9	—	17.3^{d}	18.2^{e}	21.6^{e}	19.1	—	22.0^{c}	24.7^{e}	27.2^{e}	24.6	—

Mean of three observations; PTC: pretreatment count.
In a column, means followed by a common letter(s) are not significantly different by DMRT ($P = 0.05$).

Table 2: Effect of TNAU neem oil on thrips damage in cardamom-Devarshola, Gudalur (mean of three observations).

Treatments	PTC	First round of application					Second round of application					Third round of application				
		3 DAT	7 DAT	14 DAT	Mean	% Redn	3 DAT	7 DAT	14 DAT	Mean	% Redn	3 DAT	7 DAT	14 DAT	Mean	% Redn
TNAU neem oil (A) 2%	31.0	28.6^{bc}	26.8^{b}	24.6^{b}	26.7	23.0	22.7^{bc}	20.3^{bc}	18.8^{cd}	20.6	47.0	15.3^{b}	12.8^{de}	10.9^{c}	13.0	70.6
TNAU neem oil (A) 3%	30.8	28.9^{ab}	25.1^{ab}	23.3^{ab}	25.8	25.7	21.7^{bc}	19.6^{bc}	17.9^{bcd}	19.7	49.3	13.7^{b}	8.5^{ab}	6.5^{a}	9.6	78.3
TNAU neem oil (C) 2%	29.6	27.4^{ab}	25.6^{ab}	23.2^{ab}	25.4	26.8	21.5^{bc}	19.4^{bc}	17.2^{bc}	19.4	50.2	14.3^{b}	10.2^{bc}	7.4^{ab}	10.6	75.9
TNAU neem oil (C) 3%	29.1	25.6^{a}	23.8^{a}	21.0^{a}	23.5	32.3	17.5^{a}	15.8^{a}	14.0^{a}	15.8	59.3	11.6^{a}	7.7^{a}	5.3^{a}	8.2	81.5
Neem oil 2%	29.3	29.0^{b}	27.2^{ab}	25.4^{b}	27.2	21.6	25.0^{c}	22.8^{c}	21.0^{d}	22.9	41.0	17.9^{c}	14.2^{e}	11.4^{c}	14.5	67.2
Neem oil 3%	29.9	28.3^{ab}	26.9^{ab}	24.7^{b}	26.7	23.2	22.8^{bc}	20.5^{bc}	18.9^{cd}	20.7	46.7	15.1^{b}	11.3^{cd}	9.5^{bc}	12.0	72.8
Vijay Neem 2 mL L^{-1}	30.3	27.4^{ab}	25.4^{ab}	22.9^{ab}	25.2	27.3	19.8^{ab}	17.7^{ab}	15.3^{ab}	17.6	54.7	13.8^{b}	10.9^{cd}	7.3^{ab}	10.7	75.8
Untreated check	29.3	32.7^{c}	34.7^{c}	36.7^{c}	34.7	—	37.7^{d}	38.6^{d}	40.3^{e}	38.9	—	41.7^{d}	44.2^{f}	46.4^{d}	44.1	—

Mean of three observations; PTC: pretreatment count.
In a column, means followed by a common letter(s) are not significantly different by DMRT ($P = 0.05$).

Table 3: Organoleptic evaluation of TNAU NO sprayed cardamom capsules (mean of ten scores).

Treatments	Mean scores			
	Colour	Taste	Aroma	Overall acceptability
T_1—TNAU NO (A) 3%	8.7	8.6	8.5	8.5
T_2—TNAU NO (C) 3%	8.6	8.6	8.3	8.6
T_3—control (water spray)	8.9	8.7	8.8	8.9
SED	0.200	0.226	0.206	0.207
CD (0.05)	0.410	0.464	0.422*	0.425

*Significant at 95 percent level.
SED: standard error of a difference between 2 means; CD: critical difference.

Table 4

Category	Scale
Like extremely	9
Like very much	8
Like slightly	7
Neither like nor dislike	6
Dislike slightly	5
Dislike moderately	4
Dislike very much	2
Dislike extremely	1

The data collected were subjected to analysis of variance (ANOVA) using completely randomized block design (CRD) using AGRES Version 7.01.

3.2. Field Trial II-Devarshola. The mean damage of thrips to cardamom capsules was high prior to spraying which ranged from 29.1 to 31.0 percent (Table 2). Three days after spraying, the capsule damage ranged between 25.6 and 28.9 percent in different treatments. Plots treated with TNAU NO(C) 3% registered a damage of 23.8 and 21.0 percent 7 and 14 days after treatment against 34.7 and 36.7 percent in control, respectively, and were found to be the best but not statistically superior to TNAU NO(C) 2% and TNAU NO(A) 3%. The thrips damage in TNAU NO(A) 3% treatment was on par with TNAU NO(C) 2% in all the days of observations. The maximum mean reduction in capsule damage over check of 32.3 percent was recorded in TNAU neem oil (C) at 3% followed by Vijay Neem at 2 mL L^{-1} (27.3%) at the end of first spray. Second spray was given 15 days after the first spray. Seven and 14 days after treatment, the thrips damage was found to be 15.8 and 14.0 percent in TNAU NO(C) 3% treatment, respectively, while the standard check Vijay Neem registered 17.7 and 15.3 percent, respectively. Ordinary neem oil was also somewhat effective by reducing the thrips damage, namely, from 24.7 percent before spray down to 18.9 percent, 14 days after treatment. After the third application, the thrips damage was 7.7 and 10.2 in TNAU NO(C) 3 and 2% treatments 7 days after treatment, respectively. TNAU NO(A) 3% registered 6.5 percent thrip damage 14 days after treatment while that in the standard check, Vijay Neem, was 7.3 percent. The mean reduction of thrips damage was 81.5 and 75.9 percent in TNAU NO(C) 3 and 2% sprays, respectively (Table 2). Vijay Neem registered 75.8 percent mean reduction in thrips damage when compared to control at the end of three applications.

3.3. Phytotoxicity. The treatments irrespective of the doses given did not inflict any phytotoxicity symptoms like epinasty, hyponasty, leaf injury, wilting, vein clearing, and necrosis on cardamom.

3.4. Organoleptic Tests. The mean scores graded based on the sensory perception are furnished in Table 3. There was no significant variation in the quality parameters assessed, namely, colour, aroma, taste, and overall acceptability. The standard error differences between two means of all the parameters assessed are approximately 0.2 and none of the treatments in any of the parameters evaluated are found to be statistically significant from each other.

4. Discussion

Though many chemical insecticides were reported to be effective for the management of cardamom pests [21, 22], it cannot be recommended for spraying continuously all the year. At the same time, control measures cannot be stopped because thrips will begin to infest the crop as soon as the treatment is stopped. So an effective botanical pesticide for thrips to be sprayed in between the chemical sprays can minimize the pesticide load. Particularly in the mountain ecosystem where cardamom is grown, the dislodgeable pesticides will be washed off from the plants, soil, and so forth and collected in the ponds and rivers contaminating the elixir of life—the "water." Moreover, cardamom is an export oriented crop and needs to be free of pesticide residues, and if a botanical pesticide is found to be effective, it will be an added advantage to the cardamom producers and exporters.

The extent of reduction in the thrips damage in TNAU NO(A) 3% was 31.5–76.0 percent and that of TNAU NO(C) 3% was 32.9–80.1 percent. TNAU NO(C) 2% was on par with its higher dose 3% in all the days of observations. So the two formulations were found to have no significant difference in reducing the thrips damage in cardamom. Generally, the percent reduction was low initially since the reduction of scabs in the capsules cannot be realized at once but in due course. This is evident from the continuous reduction in percent damage counts. TNAU NO(C) 3% is the best of the treatments imposed in terms of reduction in damage. The unformulated neem oil was also found effective against the thrips since it was used immediately after extraction. An overall reduction of 21% in cardamom thrips damage was reported by spraying neem oil 0.03% [16].

The reduced infestation of the cardamom pest in neem formulations sprayed field might be due to antifeedant, ovipositional deterrent or growth disturbing actions and also repellency effect. It is evident from the results that TNAU NO(C) when evaluated against different insect pests like *Cnaphalocrocis medinalis* is found to reduce the food consumption, pupal weight, adult emergence, pupation rate, and egg hatchability and to increase larval mortality [23]. The diverse biological effects of neem are also reported as it poses repellency, phagodeterrence, growth inhibition, abnormal development [24], and ovipositional suppression [25]. TNAU NO is reported as a potent ovipositional deterrent and it was found up to 90.81 percent in the laboratory. TNAU NO 0.3% causes a reduction up to 60.38 percent of thrips damage in cardamom capsules [26].

The neem sprays which were given to reduce the thrips damage and thereby to reduce the quality deterioration by the pest should not deteriorate the quality of the capsules by itself through its characteristic bitter taste or smell. Thus organoleptic test was carried out to know if there is any unacceptability for the cardamom harvested from neem sprayed field and blended with milk. Milk was taken as the medium so that any slight change in taste, aroma, or colour can be easily detected. Table 3 depicts that the scores given by the judges were between 8 and 9 which implies that the product is accepted by the consumers. This finding is in accordance with the reports of Shivashankar et al. [27], who reported no change in taste in tender coconuts harvested from soluneem (water soluble neem formulation) treated palms for the control of coconut black headed caterpillar. The present investigation clearly indicated that there are no disagreeable attributes in the harvested product of cardamom due to the application of neem which is effective in reducing the thrips and thus can be recommended for spray since it will not hamper the export also.

Conflict of Interests

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

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Review Article

Preemptive Circular Defence of Immature Insects: Definition and Occurrences of Cycloalexy Revisited

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Cycloalexy was coined by Vasconcellos-Neto and Jolivet in 1988 and further defined by Jolivet and collaborators in 1990 in reference to a specific type of circular defence. The term has been applied to numerous organisms, including adult insects, nymphs, and even vertebrates, but has lost precision with the accumulation of anecdotal reports not addressing key elements of the behaviour as first defined. We review the literature and propose three criteria that are sufficient and necessary to define the behaviour: (1) individuals form a circle; (2) defensive attributes of the individuals are positioned on the periphery of the circle, and as a result, the periphery of the circle uniformly contains either heads or abdomens; (3) animals preemptively adopt the circle as a resting formation, meaning it is not necessary to observe predation. When these considerations are taken into account, cycloalexy appears less common in nature than the literature suggests. We argue that unequivocal cases of cycloalexy have been found only in sawflies (Tenthredinoidea: Pergidae, Argidae), leaf beetles (Chrysolemidae: Galerucinae, Cassidinae, Chrysomelinae, Criocerinae), weevils (Curculionidae: *Phelypera distigma*), and midges (Diptera: Ceratopogonidae, *Forcipomyia*). Reports of cycloalexy in caterpillars (Saturniidae: Hemileucinae: *Lonomia*, Papilionidae) require further documentation. We report one new case of cycloalexy in thrips (Thysanoptera) and question reports of cycloalexic behaviour in other taxa.

1. Introduction

Some animals show a remarkable behaviour: they group in a tight circle for defence [1]. This behaviour is reminiscent of Carl von Clausewitz's 1812 Principles of War: "In strategy (...) the side that is surrounded by the enemy is better off than the side which surrounds its opponent, especially with equal or even weaker forces" [2]. Many animal species employ this strategy. For example, among vertebrates, muskoxen (*Ovibos moschatus*, Blainville, 1816) form a circle enclosing the young calves when attacked by wolves, their principal natural predators [3, 4]. Their circular formation protects the most vulnerable body parts while the extremity that is best defended or involved in attack is at the periphery. Vasconcellos-Neto and Jolivet [5] coined the term "cycloalexy (kuklos = circle, alexo = defend)" to describe a particular behaviour of gregarious insect larvae. They defined their new term as "the attitude adopted at rest by some insect larvae, both diurnal and nocturnal, in a tight circle where either the heads or ends of the abdomen are juxtaposed at the periphery, with the remaining larvae at the center of the circle. Coordinated movements such as the adoption of threatening attitudes, regurgitation, and biting, are used to repel predators or parasitoids." [1]. Several elements of the original definition distinguish cycloalexy from other circular formations occurring in nature.

However, as new examples of the behaviour have been proposed in the literature without addressing key aspects of the original definition, the distinctions between cycloalexic behaviour and other circular formations have become imprecise and weakened the concept of cycloalexy. Here, we will review reported examples of cycloalexy and question whether they meet the criteria of a revised definition or are alternative forms of aggregation. Our revised definition

strives to adhere to the key aspects of cycloalexy as originally defined, while removing arbitrary and unnecessary criteria. This way, cycloalexy can be recognized as an evolutionarily convergent behaviour rather than several superficially similar behaviours.

1.1. Redefining Cycloalexy. The defensive nature of cycloalexy is paramount as it is found in the etymology of the term: "to defend" [11]. However, this key aspect of the behaviour can be problematic since cycloalexy has often been invoked upon fragmentary observations of groups rather than controlled ecological studies. Nevertheless, until species can be more thoroughly studied, we suggest that three criteria are sufficient to distinguish cycloalexy from other behaviours.

Criterion 1: Individuals Are Arranged in a Circle. The original definition specified "in a tight circle" [1], but we argue that tightness is subjective and should be removed from the definition.

Criterion 2: The Extremity Bearing Defensive Attribute Is Positioned Outwards. In the original definition, the periphery of the circle is uniform: "either the heads or ends of the abdomen are juxtaposed at the periphery" [1], sometimes with individuals at the centre with neither head nor abdomen reaching the periphery. This means that peripheral individuals in a given group face outwards or inwards, but not both. We argue that, in this statement, it was implicit that the best defended extremity is at the periphery since it is unlikely that individuals with their vulnerable side out could ever be at an advantage. Uniformity of the circle in this respect, then, becomes a corollary of our second criterion.

Criterion 3: The Circle Is Adopted as a Resting Formation. Following the original definition, we limit cycloalexy to cases when individuals are in resting or quiescent, nonfeeding periods. This makes cycloalexy a preemptive behaviour.

These criteria allow for the initial identification of cycloalexy by rapid, visual assessment. If later studies disprove defensiveness, then the behaviour studied is not cycloalexy. Additionally, although not specified in the original definition, we suggest adding the distinction that the behaviour is for the defence of the individuals themselves and others within the group, as opposed to the defence of a resource or nest. We also suggest removing the criterion that "Coordinated movements such as the adoption of threatening attitudes, regurgitation, and biting, are used to repel predators or parasitoids" [1], for several reasons. Cycloalexy is notably preemptive, taken regardless of the immediate presence of a threat; in some species, immature animals have passive defences made more efficient by a circular formation; and the second criterion of our amended definition already includes defence, either passive or active. Finally, although the original definition was limited to insect larvae, in this paper, we review all reports of cycloalexy and suggest removing the limitation altogether.

2. Results

See Table 1.

3. Discussion

3.1. Strict Cycloalexy. Among the records in the literature, some species demonstrate behaviour that precisely fit the revised definition and our three essential criteria of cycloalexy (Table 1). This is the case of *Coelomera* spp. (Coleoptera: Chrysomelidae: Galerucinae) and spitfire grubs *Perga dorsalis* Leach, 1817 (Hymenoptera: Tenthredinoidea: Pergidae) [1].

Approximately 35 species of genus *Coelomera* are cycloalexic and feed on *Cecropia* (Urticaceae). Most *Cecropia* plants are myrmecophytes protected by mutualistic *Azteca* ants (Formicidae: Dolichoderinae). The gregarious leaf beetle larvae feed during the day and rest at night, in a tight circular cluster with heads inside and abdomens at the periphery [7, 8]. Their rear end is protected by a supra-anal shield and, when threatened, these larvae excrete a nauseating fluid from the anus. Therefore, the better protected part of the insect, the posterior, is facing outwards in the circle, with the more vulnerable head inside [7, 8]. Thus, by orienting the same way, individual larvae protect themselves and other members of the group (Figures 1 and 2).

Spitfire grubs *Perga dorsalis* feed on *Eucalyptus* during the night and rest during the day in a circular formation [47]. The larvae rest with their heads at the periphery of the circle, with some larvae in the middle of the aggregation. When threatened, the larvae rear their heads and abdomens and regurgitate oils sequestered from their host *Eucalyptus* [47]. The oils are an effective deterrent of potential predators, including ants, birds, and mice [49]. The heads are the better protected part of the insects and, again, form the periphery of the circle.

3.2. Examples of Cycloalexy That Do Not Agree with the Revised Definition

3.2.1. The Oxymoron of Noncircular Cycloalexy. Gregarious caterpillars of genus *Arsenura* (Saturniidae: Arsenurinae) are reported to "show a kind of cycloalexy when resting on tree trunks during the day" [1]. The caterpillars align side-by-side or head-to-abdomen or both, in an elongated oval cluster [11]. The posture of these caterpillars with their heads, sides, and abdomens at the periphery in a linear mass rather than a circle does not meet the first criterion of the revised definition of cycloalexy. The circle formation with the best defended extremity outwards is an important characteristic of cycloalexic behaviour. *Arsenura* are gregarious and rest in a tightly aggregated mass, but they are not cycloalexic. Santiago-Blay et al. [11] suggest that, on a tree trunk, "the available background surface makes the shape of the larval aggregation distorted." However, on the scale of a caterpillar, and depending on the diameter of the tree, a tree trunk can be quite large and nearly flat. In addition, probable cycloalexy on tree trunks has been observed in *Lonomia* sp. (Figure 3(b)). We suggest the caterpillar aggregations described by Santiago-Blay et al. [11] are less circular and compact not because of the shape of tree trunks but because *Arsenura* caterpillars' resting positions are not cycloalexic.

Table 1: Reported cases of cycloalexy and how they fit within the revised definition.

Organism	Criteria 1 Circular formation	2 Best defended extremity at the periphery	3 Default rest position	Aggregation is defensive (not for nest protection)	Is the behaviour cycloalexy?	Reference
Coleoptera: Chrysomelidae: Galerucinae						
Coelomera spp.; for example, *C. ruficornis* Baly, 1865; *C. helenae* Jolivet, 1987; *C. raquia* Bechyně, 1956; and so forth	Yes	Yes, abdomens	Yes	Yes	Yes	[1, 5–8]
Dircema spp.	Not observed	Not observed	Not observed	Not observed	None observed	[6, 7]
Coleoptera: Chrysomelidae: Criocerinae						
Lema sp.; *Lema apicalis* Lacordaire, 1845 and *L. reticulosa* Clark, 1866	Yes	Yes, heads	Yes	Yes	Yes	(Figure 1(a)) [9, 10]
Lilioceris nigropectoralis (Pic, 1928), *L. formosana* Heinze, 1943	Yes	Yes, heads	Yes	Yes	Yes	(Figure 1(b)) [11]
Coleoptera: Chrysomelidae: Chrysomelinae						
Agrosteomela chinensis (Weise, 1922)	Not observed	Not observed	Not observed	Not observed	None observed	[11–13]
Chrysophtharta obovata (Chapuis, 1877)	Yes	Yes, abdomens	Yes	Probably	Yes	[11, 14]
Doryphora paykulli (Stål, 1859), *D. reticulata* Fabricius, 1787	Yes	Unclear, abdomens	Yes	Yes	No*	[15]
Eugonycha melanostoma (Stål, 1859)	Yes	Unclear, abdomens	Not reported	Not reported	Tentatively	[7, 11]
Gonioctena sibirica Kimoto, 1994	Roughly	Unclear, mostly abdomens	Mostly	Unclear	Unclear	[11, 12, 16]
Labidomera suturella Guérin-Méneville, 1838	Not observed	Not observed	Not observed	Not observed	None observed	[11, 17–19]
Paropsis spp.; for example, *P. aegrota* Boisduval, 1835, *P. maculata* (Marsham, 1908), *P. atomaria* Olivier, 1807 and *P. tasmanica* Baly, 1864	Not circular	No, mixed extremities	No	Perhaps, unclear	No	[7, 11, 14, 20, 21]
Paropsisterna spp.	Not reported	Not reported	Not reported	Not reported	Not enough information	[11, 12, 14, 20]
Plagiodera spp. for example, *P. versicolora* (Laicharting, 1781)	Not circular	No, mixed extremities	No	No	No	[7, 22–25]
Phratora spp.	Not observed	Not observed	Not observed	Not observed	None observed	[7, 11]
Phyllocharis undulata (Linnaeus, 1763)	Roughly	Unclear, mostly abdomens	No	Not observed	No	[11, 26]
Platyphora selva Daccordi, 1993, *P. microspina* (Bechyně, 1954)	Yes	Unclear, abdomens	Yes	Yes	No*	[15, 27]
Platyphora conviva (Stål, 1858), *P. anastomozans* (Perty, 1832), *P. nigronotata* (Stål, 1857), *P. nitidissima* (Stål, 1857) *P. fasciatomaculata* (Stål, 1857), *P. vinula* (Stål, 1858)	Yes	Yes, heads	Yes	Yes	Yes	[7, 9, 10, 28]

Table 1: Continued.

Organism	Criteria 1 Circular formation	2 Best defended extremity at the periphery	3 Default rest position	Aggregation is defensive (not for nest protection)	Is the behaviour cycloalexy?	Reference
Proseicela vittata (Fabricius, 1781), *P. bicruciata* Jacoby, 1880, *P. spectabilis* (Baly, 1858)	Yes	Unclear, abdomens	Yes	Yes	No*	(Figure 5(a)) [15]
Proseicela crucigera (Sahlberg, 1823)	Yes	Unclear, abdomens	Yes	Yes	Not enough information	[7, 9]
Pterodunga mirabile Daccordi, 2000	Yes	Unclear, abdomens	Not reported	Not reported	Not enough information	[11, 12, 19]
Coleoptera: Chrysomelidae: Cassidinae						
Acromis sparsa (Boheman, 1854)	Yes	Yes, abdomens	Yes	Yes	Yes*	[7, 11, 29]
Aspidomorpha puncticosta Boheman, 1854, *A. miliaris* (Fabricius, 1775)	Yes	Yes, abdomens	Yes	Yes	Yes	[7, 30–32]
Chelymorpha informis Boheman, 1854, *C. alternans* Boheman, 1854, *C. cribraria* (Fabricius, 1875)	Yes	Yes, abdomens	Yes	Yes	Yes	[7, 8]
Cistudinella foveolata (Champion, 1894)	Yes	Yes, abdomens	Yes	Probably	Yes	(Figure 2(a))
Conchyloctenia punctata (Fabricius, 1787)	Yes	Yes, abdomens	Yes	Yes	Yes	[7, 30, 33]
Coptocycla dolosa Boheman, 1855	Yes	Yes, abdomens	Yes	Probably	Yes	(Figure 4)
Eugenysa columbiana (Boheman, 1850), *E. coscaroni* Viana, 1968	Yes	Yes, abdomens	Yes	Yes	Yes*	(Figure 2(b)) [34, 35]
Paraselenis flava (Linnaeus, 1758)	Yes	Yes, abdomens	Yes	Yes	Yes*	[8]
Nuzonia sp.	Yes	Yes, abdomens	Yes	Yes	Yes	(Figure 2(c))
Ogdoecosta biannularis (Boheman, 1854)	Yes	Yes, abdomens	Yes	Yes	Yes	[7, 11, 36]
Omaspides tricolorata (Boheman, 1854), *O. pallidipennis* (Boheman, 1854), *O. sobrina* (Boheman, 1854), *O. bistriata* (Boheman, 1854) and *O. convexicollis* Spaeth, 1909	Yes	Yes, abdomens	Yes	Yes	Yes*	[7, 11, 32, 35, 37–39] (D. Windsor's observations)
Physonota alutacea Boheman, 1854	Yes	Yes, abdomens	Yes	Probably	Yes	(Figure 2(d))
Polychalma multicava (Latreille, 1821)	Yes	Yes, abdomens	Yes	Probably	Yes	(Figure 2(e))
Stolas sp., *Stolas xanthospila* (Champion, 1893)	Yes	Yes, abdomens	Yes	Probably	Yes	(Figure 2(f)) [7]
Coleoptera: Curculionidae: Hyperinae						
Phelypera distigma (Boheman, 1842)	Yes	Yes, heads	Yes	Yes	Yes	[24, 40, 41]
Diptera: Ceratopogonidae: Forcipomyiinae						
Forcipomyia fuliginosa (Meigen, 1818)	Yes	Yes, abdomens	Yes	Probably	Yes	[1, 11, 42–44]
Hemiptera						
Not specified	Not reported	Not reported	Not reported	Not reported	Not enough information	[11, 12]
Ceroplastes sp. (Coccidea), *Potnia* sp. (Membracidae), *Nephesa rosea* (Spinola, 1839) (Flatidae), *Derbe* sp. (Derbidae)	Roughly to not circular	No, mixed extremities	No	Unclear	No	[11]
Antiteuchus tripterus (Fabricius, 1787) (Pentatomidae)	Yes	Unclear, abdomens	No	No	No	[45]
Parastrachiajaponensis (Scott, 1880) (Parastrachiidae)	Yes	Unclear, abdomens	No	No	No	[46]

Table 1: Continued.

Organism	Criteria 1 Circular formation	2 Best defended extremity at the periphery	3 Default rest position	Aggregation is defensive (not for nest protection)	Is the behaviour cycloalexy?	Reference
Hymenoptera: Tenthredinoidea						
Bergiana sp. (Cimbicidae)	Yes	Not reported	Not reported	Not reported	Not enough information	[1]
Perga dorsalis Leach, 1817, *P. affinis* Kirby, 1882 (Pergidae)	Yes	Yes, heads	Yes	Yes	Yes	(Figure 3(a)) [7, 11, 12, 47, 48]
Pseudoperga guerini (Westwood, 1880) (Pergidae)	Yes	Yes, heads	Yes	Yes	Yes	[49]
Themos olfersii (Klug, 1834) (Argidae)	Yes	Yes, heads	Yes	Yes	Yes	[1, 50]
Dielocerus diasi Smith, 1975 (Argidae)	Not reported	Not reported	Not reported	Unclear	Not enough information	[1, 50]
Hymenoptera: other superfamilies						
Trigona sp. (Apidae: Meliponinae)	Yes	Yes, heads	No	No	No	[1, 7, 11, 51]
Adult Hymenoptera, bees (Apidae), wasps (Vespidae), *Conomyrma* spp. and numerous other ants (Formicidae)	Some circular, some not	Yes, usually heads	No	No	No	[11]
Apoica sp. (Vespidae: Polistinae)	Yes	Yes, heads	Yes	No	No	[52–54]
"Parasitic Hymenoptera larvae and pupae [on] their host"	Yes	Unclear, abdomens	No	No	No	[11]
Lepidoptera: Papilionidae: Papilioninae						
Papilio laglaizei Depuiset, 1877	Yes	Unclear, heads	Yes	Not reported	Tentatively	[24, 55]
Lepidoptera: Saturniidae						
Hylesia spp. (Hemileucinae)	Unclear	Not reported	Yes	Probably	Not enough information	[7]
Lonomia spp. (Hemileucinae)	Yes	Probably, heads	Yes	Probably	Probably	(Figure 3(b)) [11, 56, 57]
Arsenura spp. (Arsenurinae)	Not circular	No, mixed extremities	Yes	Probably not	No	[1, 11].
Lepidoptera: other families						
Noctuidae and Sphingidae	Not circular	No, mixed extremities	Not reported	Probably not	No	[11]
Neuroptera: Ascalaphidae						
Ascaloptynx furciger (McLachlan, 1891)	Yes, around twig	No, mixed extremities	No	Yes	No	[1, 11, 12, 58]
Thysanoptera: Phlaeothripidae						
Anactinothrips nigricornis Hood, 1936 and *A. gustaviae* Mound & Palmer, 1983	Yes	Yes, abdomens	Yes	Probably	Yes	(Figure 5(b)) [59]
Non-insect arthropods						
Phronima sedentaria (Forskål, 1775) (Crustacea: Amphipoda: Hyperiidea)	Yes	Unclear, heads?	Yes	No	No*	[24, 60, 61]
Platydesmidae, Unidentified sp. (Myriapoda)	Yes	Unclear, abdomens	Not reported	Not reported	Tentatively analogous	[24, 62]
Vertebrates						
Some ungulates, for example, Muskoxen *Ovibos moschatus* (Zimmermann, 1780)	Yes	Yes, heads	No	Yes	No	[1, 11, 12]
Antarctic penguins	Yes	Unclear, backs	Yes	No	No	[12, 63]

*These taxa are maternally defended and pose a special challenge to the definitions of cycloalexy (see Section 3.2.5).

(a) (b)

FIGURE 1: Cycloalexywith heads outwards in shining leaf beetle larvae (Criocerinae). (a) Larvae of *Lema* sp. at rest, photograph in Potrerillos del Guendà, Dept. Santa Cruz, Bolivia, © D. Windsor. (b) *Lilioceris nigropectoralis* larvae in Taiwan. Seven larvae are distinctly larger and appear to be from a different cohort than the other twelve. Photograph taken in Yangmingshan National Park on 2 August 2011, by 劉達偉 (Liu Dá Wěi), and licensed under the Creative Commons 3.0 Taiwan (CC BY-NC 3.0 TW).

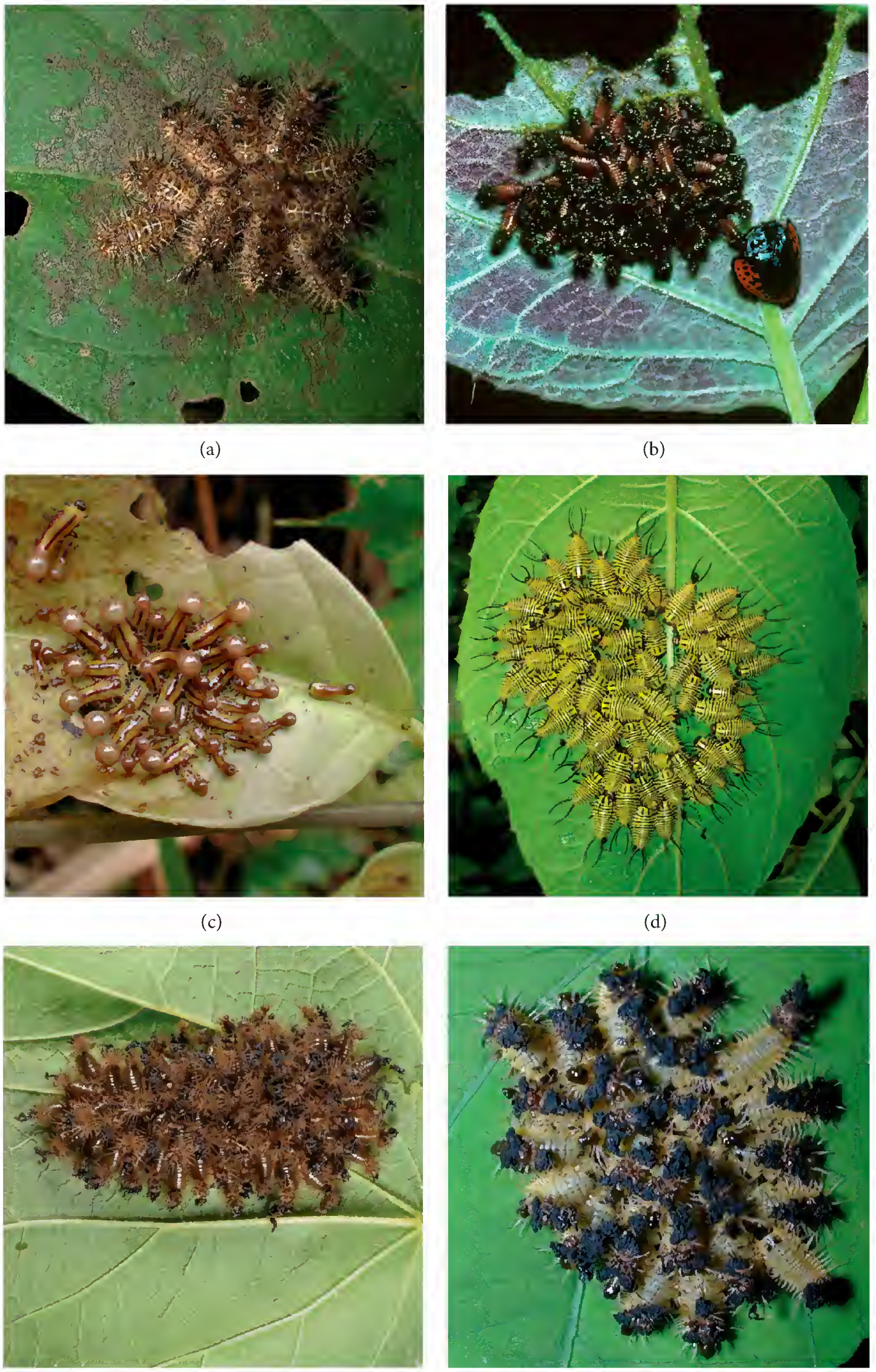
3.2.2. Mixed Head Orientations. Larvae of the owlfly *Ascaloptynx furciger* (McLachlan, 1891) (Neuroptera: Ascalaphidae) are gregarious. After eclosion and their first meal of abortive eggs, they settle head-downwards on and around the twig on which they were laid [58]. Jolivet et al. [1] deem the behaviour of *A. furciger* is "not strictly cycloalexy but related to it" since the owlfly larvae all point in the same downward direction: this does not meet the second criterion of the revised definition. We agree with Jolivet and Verma [12] that cycloalexy exists around twigs and is not restricted to flat surfaces. However, even on small branches, cycloalexic larvae collectively orient their heads either outwards or inwards, but not both. This is true for larvae of *Perga* sp. (Hymenoptera: Tenthredinoidea: Pergidae) (Figure 3(a)), *Omaspides tricolorata* (Boheman, 1854) [39], and this arrangement is retained in the pupae of *Omaspides pallidipennis* (Boheman, 1854) (Chrysomelidae: Cassidinae) [37]. For owlfly larvae, heads form the periphery at the bottom of the aggregation and abdomens are at the periphery on top, but unlike Cassidinae or *Coelomera* larvae, their abdomens are more vulnerable. It is more accurate to describe the behaviour as unidirectional defence rather than circular defence; larvae are only protected from predators walking up to the group. Secondly, larvae also feed while, in this position, making it a passive hunting formation and not only a resting position [58]. Because they do not meet the second and third criteria, we question reports of cycloalexy in Neuroptera [7, 11, 12, 64].

3.2.3. Nonresting Behaviours. As stated in the third criterion, cycloalexy is adopted preemptively by animals at rest. When immature insects are active and feeding, the circular formation is normally broken (Figure 4). Larvae of *Plagiodera versicolora* (Laicharting, 1781) and other *Plagiodera* species form a loose circle when feeding and at rest, with individual larvae not consistently facing outwards or inwards [22, pers. obs.]. Hence their formation is not an example of cycloalexy. Their formation is not only adopted at rest but also while feeding and is often influenced by the shape of the leaf, with multiple "feeding rings" on larger leaves [22]. Some authors [11] feel that cycloalexy facilitates feeding in *P. versicolora* as well as in sawflies. Larval aggregations can increase feeding efficiency through synchronized, coordinated, and spatially concentrated feeding [23–25].The size of *P. versicolora* groups does not influence survival of larvae, but does help with feeding [25]. Thus, available evidence suggests grouping in *P. versicolora* is related principally to the process of feeding rather than defence.

3.2.4. Nondefensive Behaviour. Cycloalexy is a defensive behaviour; it protects individuals from predation or parasitism. Yet, some reported behaviours are not defensive. Such is the case for huddling in Antarctic penguins, where the huddle is a resting behaviour, usually with heads inwards, but it is for heat conservation rather than defence [63]. For these reasons, we disagree with Jolivet and Verma [12] that penguins are cycloalexic.

To conclusively prove the defensive value of a behaviour, ecological studies are needed. Yet, for many species, the defensive value of cycloalexy has been inferred from anecdotal evidence or personal observations or has simply been presumed. For example, the defensive value of cycloalexy in *Phelypera distigma* larvae is supported by the following statement: "*P. distigma* larvae are not harvested by polistine wasps, ants, spiders, and other generalist predators that readily harvest caterpillars in dry forest habitats (D. H. Janzen, pers. obs.)" [65].

Rather than rejecting the many reports of cycloalexy on the basis of insufficient ecological studies, we propose that defensive nature of the aggregation can be accepted if the



(a) (b) (c) (d) (e) (f)

FIGURE 2: Cycloalexy in tortoise beetle larvae. (a) *Cistudinella foveolata* (Ischyrosonychini) larvae on host *Cordia alliodora* (Ruiz & Pav.) Oken. Gamboa, Colón Province, Panama; (b) *Eugenysa coscaroni* (Eugenysini) larvae and mother on host *Mikania guaco* Bonpl. (Asteraceae), Cerro Campana, Panama Province, Panama; (c) *Nuzonia* sp. on host *Maripa nicaraguensis* Hemsl., Chiriquí Grande, Bocas del Toro Province, Panama; (d) *Physonota alutacea* (Ischyrosonychini) larvae on host *Cordia spinescens* L., Gamboa, Colón Province, Panama; (e) *Polychalma multicava* (Goniocheniini) larvae on host *Helicteres guazumaefolia* Kunth. (Sterculiaceae), Gamboa, Colón Province, Panama; (f) *Stolas xanthospila* (Mesomphaliini) larvae on host *Turbina corymbosa* (L.) Raf. (Convolvulaceae), Cerro Campana, Panama Province, Panama; all photographs © D. Windsor.

(a) (b)

FIGURE 3: (a) Larvae of *Perga* sp. (Pergidae) rest aggregated in a cycloalexic formation. Even around a stem of their host plant, *Eucalyptus* sp., spitfire larvae rest with their heads outwards, Black Mountain, Canberra, ACT, Australia. Photograph by Donald Hobern on 24 May 2010 (CC BY 2.0). (b) Probable cycloalexy with heads pointing outwards in caterpillars of *Lonomia* sp. (Saturniidae: Hemileucinae) on tree trunk in Peru. Photograph taken near Pongo de Caynarachi, Lamas, San Martin, Peru, and reproduced with the author's permission © Marc Díaz Rengifo (Universidad Nacional Federico Villarreal, Lima, Perú).

(a) (b)

FIGURE 4: *Coptocycla dolosa* larvae, Potrerillos del Guendà, Dept. Santa Cruz, Bolivia. (a) when active, feeding, or moving; (b) at rest. © D. Windsor.

animals meet the other criteria: they are in a circle, taken preemptively with defensive armature uniform at the periphery. When ecological studies are conducted, if defensiveness is disproved, then the behaviour is another type of aggregation and not cycloalexy. This is the case for larval aggregations of *Plagiodera versicolora*: ecological studies were conducted and the survival of larvae is not significantly influenced by group size [25].

3.2.5. Circular Formations That Do Not React to Threats. The original definition of cycloalexy requires coordinated movements in response to threats [1]. We disagree with this requirement: in some larvae with passive protection, like the exuvial or exuvio-fecal shields of tortoise beetles, the circular groups do not always use coordinated movements when threatened by predators. For example, the larvae of *Conchyloctenia punctata* (Fabricius, 1787) (Cassidinae) are passively protected by their shields but do not have coordinated reactions to threats [30]. In our opinion, larvae of *C. punctata* meet the basic criteria of cycloalexy. Although coordinated group reactions to threats are an indication of the defensive nature of the group, we propose it is not an essential criterion for cycloalexy.

The removal of this criterion is also important for several taxa in which the larvae receive maternal care. Cassidinae larvae in maternal care species (e.g., species of genera *Acromis*, *Omaspides*, *Paraselenis,* and *Eugenysa*) generally have reduced fecal shields and do not always react defensively

when threatened. Larval grouping in these species can be considered as increasing the efficiency of maternal guarding. In these cases, all criteria of the revised definition are met: larvae are in a circle, the best defended extremity is always at the periphery, and the circle is the default resting position. Thus, we consider larval aggregations in these maternal care species as further examples of cycloalexy (Table 1).

Similarly, larvae of several chrysomelines rest in tight circular groups with the heads pointing inwards: *Doryphora paykulli* (Stål, 1859), *D. reticulata* Fabricius, 1787, *Platyphora microspina* (Bechyně, 1954), *P. selva* Daccordi, 1993, *Proseicela vittata* (Fabricius, 1781), *P. spectabilis* (Baly, 1858), *P. bicruciata* Jacoby, 1880 and *Pr.* sp. nov. "Yasuni" [15]. All these species also have maternal care, and when disturbed, larvae do not have coordinated defensive reactions. Instead, the mother acts as the defensive element of the formation (Figure 5(a)) [15]. Is this behaviour still cycloalexy? In other words, should the defensive element obligatorily be found, at least in part, in the larvae? To this question, our answer is yes, through the second criterion. In Cassidinae larvae, the furca and shield are obvious defensive attributes positioned at the periphery. In Chrysomelinae, the best defended extremity is less obvious. Cycloalexic larvae of nonmaternal care Chrysomelinae face outwards. Their best defended extremity is the head and thorax, through regurgitation and biting [7]. We hypothesize that, in species with maternal care, the individuals face inwards not because the best defended extremity is the abdomen but because of herding by the mother, and thus, these species do not meet the second criterion of the revised definition. Ultimately, only ecological and evolutionary studies will provide a clear answer.

3.2.6. Adult Insects. We use *Apoica* as an example even though cycloalexy was not explicitly reported in this genus. During the day, these nocturnal wasps rest on the circular or nearly circular lower surface of their nests [52–54]. The wasps rest facing outwards, resulting in a circular formation that could loosely be termed cycloalexy. When disturbed, the formation breaks up as wasps fly off the nest. Even though this behaviour meets several criteria of the revised definition, we argue it is not cycloalexic because the shape of the nest or nest entrance explains the circular formation. In a similar fashion, stingless bees of genus *Trigona* (Apidae: Meliponinae) are not cycloalexic as suggested by Vasconcellos-Neto and Jolivet [7]. In this case, fully developed individuals are not even at rest: in most Meliponinae, the nest entrance is protected by bees positioned in or around the entrance tube and, at night, the entrance is closed [51]. The bees are not resting but are actively guarding and the ring formation is an artefact of the nest entrance shape.

These examples motivate limiting and specifying cycloalexy as a formation taken by individuals, whether immature or adult, for increased individual and mutual defences, thus excluding formations taken for defence of a nest, brood, or food stores. We argue that evolution of circular nests and resource guarding may have little to do with the evolution of cycloalexy.

3.2.7. Circular Defence in Vertebrates and the Selfish Herd. Several authors compare cycloalexy to the "circle-the-wagons" formation employed by American pioneers to defend themselves against Native Americans [1, 11, 12, 24]. In Jolivet et al. [1] and Jolivet and Verma [12], the authors discuss behaviours analogous to cycloalexy in vertebrates: muskoxen (*Ovibos moschatus*), eland (*Taurotragus oryx* (Pallas, 1766)), elk (*Cervus canadensis* (Erxleben, 1777)), and penguins. The authors do not provide citations for the behaviour in eland or elk and cite Wilson [4] for descriptions of this behaviour in muskoxen and penguins. Wilson [4] does not mention penguins in this manner but does mention similar behaviours in several terrestrial ungulates and killer whales (*Orcinus orca* (Linnaeus, 1758)) ([3, 66–69]; all page 45 in [4]). Wilson [4] describes elk grazing in a "windrow" formation but does not mention circular defence [70] and [4, page 45]. We agree that several vertebrates employ defensive circular formations analogous to cycloalexy. However, we would not broaden the definition to include these behaviours. Unlike invertebrates, mammals do not use circular defence when resting but take the formation when threatened. This does not meet the third criterion of the revised definition. In cycloalexic species, the circular formation is the main resting position. The circular defence of vertebrates is reactive, while cycloalexy in invertebrates is largely preemptive.

Hamilton used herding animals as an example of how individuals may form a group to lessen individual chances of falling to a predator without reducing overall predation [71]. Hamilton then cited the circular defence of muskoxen as a potential exception to the selfish herd theory but attributes it to selfish reasons: "they are probably connected on the one hand with the smallness of the risk taken and, on the other, with the closeness of the genetical relationship of the animals benefited" [71]. Because cycloalexy may lessen both overall and individual predation risk, it can also be considered selfish. Cycloalexy can be explained by animals exploiting the best defended extremity of nearby individuals. The preemptive aspect of arthropod cycloalexy also distinguishes it from muskoxen circular defence and Hamilton's selfish herds and may therefore provide interesting systems for study of group defence.

3.2.8. Cycloalexy in Immature Hemimetabolous Insects. We report cycloalexy in *Anactinothrips nigricornis* Hood, 1936 (Thysanoptera). We observed a group of 14 thrips, in their pupal instar, forming a tight circle with abdomens outwards on a leaf of the woody vine *Maripa panamensis* Hemsl. (Convolvulaceae) (Figure 5(b)). When disturbed, the threatened individuals and those beside them waved their abdomen. When disturbance continued, a brown liquid was exuded and formed a droplet at the end of the abdomen. The group was then further disturbed and the individuals dispersed. Approximately an hour later, the thrips had reassembled in a circular resting formation. In the lab, after the final moult, the adult thrips dispersed in the container in which they were kept. Similar observations were made in another species of the same genus: the thrips *A. gustaviae,* Mound and Palmer,

(a) (b)

FIGURE 5: (a) Larvae of *Proseicela spectabilis* Baly (Chrysomelidae: Chrysomelinae) at rest encircling the stem of their host plant with tachinid fly at the bottom left of the cluster and the adult female beetle on the opposite side. Photograph taken in Reventador, Napo Province, Ecuador, © G. Dury. (b) Circular resting position in Panamanian thrips (*Anactinothrips* sp.) on *Maripa panamensis* Hemsl. (Convolvulaceae). Photograph taken 24 April 2013, on Cerro Campana, Panama. © G. Dury.

1983, rest in bivouacs and exude a defensive liquid from their abdomen when disturbed [59, 72].

The behaviour was observed in the mobile pupal stage rather than in the larvae. This goes against the original definition but meets all other criteria and assuming it is defensive, we consider the behaviour is cycloalexy. Thus, we propose to remove the taxonomical restriction of the original definition.

3.3. Common Traits of All Cycloalexic Species. When the revised definition of cycloalexy is strictly applied, a set of traits common to all species becomes apparent. Foremost, all cycloalexic species are insects with gregarious immature stages. Gregarious lifestyles have implications in terms of cooperative feeding and continued group cohesion through chemical, tactile, or acoustic communication [24].

To date, all cycloalexic species appear to use chemical defences of one sort or another. The cycloalexic larvae in genera *Lema* (Criocerinae) and *Platyphora* (Chrysomelinae) regurgitate when threatened [7, 9]. The larvae of *Forcipomyia* have paired setae on the head, thorax, and abdomen that exude hygroscopic substances that repel ants [44]. The chemical defences of gregarious *Lonomia* caterpillars are so potent that the resulting trauma caused by venom injected from their setae can be lethal to humans [73]. Most tortoise beetle larvae carry an exuvial or exuvio-fecal shield on the furca of their eighth abdominal segment which serves as a mechanical or chemical barrier against predators [74–76]. In all cases, the best protected extremity faces outwards.

Furthermore, all the species that exhibit cycloalexic behaviour are miniature grazers, and most feed on leaves. This is the case for cycloalexic caterpillars, and larvae of sawflies [47], weevils, and leaf beetles [7, 40]. Some feed on fungal hyphae, such as *Forcipomyia fuliginosa* (Meigen, 1818) midge larvae [42], and the rest graze on lichen, like *Anactinothrips gustaviae* thrips [59].

Gregarious lifestyles, chemical defence, and grazing groups of immature insects are all traits of Costa's [24] "larval herd" syndrome of group living. Like cycloalexy, parental care is only present in some of these larval herds [24]. Possibly, the slow-moving and exposed lifestyle of these immature insects makes them more vulnerable to predators and parasitoids [24, 77]. Increased threats probably explain the multiple defences of insect herbivores, including chemical defence whose evolution generally precedes that of aggregation [78].

4. Conclusion

Several immature insects exhibit cycloalexy, a behaviour whose definition we have amended to: "A preemptive defence employed at rest, where individuals form a circle with their best defended extremity exposed at the periphery. Sometimes remaining individuals rest at the centre of the circle."

In leaf beetles (Chrysomelidae), cycloalexy with abdomens oriented outwards is found in one genus of skeletonizing leaf beetles (Galerucinae: *Coelomera* spp.), at least fifteen tortoise beetle genera (Cassidinae), two genera of shining leaf beetles (Criocerinae: *Lema* and probably *Lilioceris*), and several genera of broad-shouldered leaf beetles (Chrysomelinae: *Platyphora*, probably *Chrysophtharta* and tentatively *Eugonycha* and *Pterodunga*). Cycloalexy with heads outwards is found in some sawflies (Tenthredinoidea: Pergidae: *Perga* spp. and Argidae: *Themos olfersii* (Klug, 1834)) of Australia and Brazil. Social caterpillars often form aggregations, but

these aggregations are rarely cycloalexic. However, caterpillars of *Lonomia* spp. (Saturniidae: Hemileucinae) are probably cycloalexic and *Papilio laglaizei* Depuiset, 1877 (Papilionidae) are tentatively cycloalexic. One weevil *Phelypera distigma* (Boheman, 1842) (Curculionidae) is cycloalexic and one midge *Forcipomyia fuliginosa* (Ceratopogonidae) exhibits cycloalexy. We propose that some immature thrips are also probably cycloalexic and suggest formally changing the definition of cycloalexy to remove taxonomical restrictions so that any animals that meet all other criteria of the definition can be included. New instances of cycloalexy will undoubtedly be discovered. For example, Platydesmid millipedes sometimes aggregate in a tentatively analogous fashion.

Several reports of cycloalexy do not meet one or more of the revised definition criteria, including reports of cycloalexy in feeding aggregations of Hemiptera and larvae of Hymenopteran parasitoids. The behaviour has also been mistakenly attributed to adult Hymenoptera, for example, stingless bees (Apidae: Meliponinae), ants (Formicidae), and wasps (Vespidae), guarding their nest. This is active protection of a nest and not cycloalexy. Similarly, the term has been applied to the circular assembly of an amphipod crustacean which helps the mother herd the larvae. Owlfly larvae (Neuroptera: Ascalaphidae: *Ascaloptynx furciger*) form unidirectional defensive groups which are not cycloalexic, allowing larvae to feed without changing position. Defensive circles are sometimes observed in mammals: muskoxen, eland, water buffalo, red deer, and killer whales. Contrary to cycloalexy, the defensive formations in these mammals are a reaction to imminent threat. Other vertebrates, like penguins, huddle to reduce heat loss.

Application of a more precise definition of cycloalexy, as provided by Jolivet et al. [1] and revised here, may make unravelling the evolution of cycloalexic behaviour more tractable. Much remains to be learned about whether larval aggregation, cycloalexy, sequestration of plant metabolites, and maternal care are alternative defensive strategies or are honed evolutionary responses to particular threats. Chrysomeline leaf beetles are an ideal group for using phylogenetic reconstruction and character analysis of these behaviours to unravel the number of independent evolutionary origins of cycloalexy and larval aggregation.

Conflict of Interests

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

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Research Article

Prospects for the Use of *Pongamia pinnata* Oil-Based Products against the Green Peach Aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae)

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This study is devoted to an estimation of the action of preparations based on *Pongamia pinnata* oil on the life cycle (survival, fecundity) of green peach aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). The *M. persicae* is a widespread pest and damages more than 100 species of plants. All test formulations had aphicidal activity for *M. persicae* adults and larvae. Moreover, they possess prolonged action, exerting a negative influence on the offspring. The preparations differed in speed of onset of mortality. The single treatment with these formulations provides significant reduction in the number of aphids during the observation period, because of the efficiency rising in time.

1. Introduction

A wide range of chemicals are used to protect plants from pests in modern agriculture. Most of them are not destroyed by enzyme systems of plants, or by external natural impacts, that is cause their accumulation in the crop, and as a result—in humans and animals. Regular treatments cause the occurrence of resistance in harmful objects and, at the same time, are dangerous for beneficial arthropods.

One possible solution to these problems is the search for new active substances—secondary plant metabolites. The ability of plants to produce antibiotic substances is well known [1, 2]. Currently, the list of known plant secondary metabolites continues to expand. Enough information to support the use of plant preparations for crop protection is already accumulated [3, 4].

Botanical insecticides are natural chemical substances isolated from plants. Such preparations may be considered as an alternative to synthetic chemical compounds, but they are not always less toxic to mammalian. But botanical insecticides are easily decomposed in the soil and will not be stored in the tissues of plants and animals.

Among the substances of plant origin, essential oils, which have well-known activity against various pests, occupy a special place. So, essential oils from plants of the *Mentha* genus, *Origanum vulgare* L., *Ocimum basilicum* L. (Lamiaceae), and *Carum carvi* L. (Umbelliferae) are highly toxic to *Trialeurodes vaporariorum, Tetranychus cinnabarinus, Acanthoscelides obtectus,* and *Meligethes aeneus* [5–8].

One of the most interesting objects of study in recent years is Pongamia oil, the use of which is widespread in various areas of activity. According to the literature and earlier results, this plant has insecticidal activity to some pest and synanthropic insects [9, 10].

Myzus persicae (Sulzer) (Hemiptera: Aphididae) is a widespread polyphagous aphid, which damages more than 100 species of plants. In greenhouses, there are forms with incomplete development cycle that can reproduce continuously throughout the year [11, 12]. Pesticide with broad spectrum is not effective enough due to the high resistance of *M. persicae* to organophosphate and pyrethroid insecticides [13].

With year-round growing season, the problem of the replacement of traditional pesticides with new means of pest

control is actual in greenhouse crop production, where there is a constant accumulation of harmful arthropods.

The aim of our work is to evaluate the action of preparations based on *Pongamia pinnata* (L.) Pierre oil on the life cycle (survival, fecundity) of green peach aphid.

2. Materials and Methods

2.1. Insects. The green peach aphid *Myzus persicae* (Sulzer) was reared on sprouting broad beans (*Vicia faba* L.), grown at 22 ± 2°C, 16 : 8 LD, and 50 ± 10 RH.

2.2. Plant Material and Chemicals. Pongam is seed oil from *Pongamia pinnata* (Parker Group, India) emulsified using Tween 85. The oil was tested by HPLC to determine the Karanjan content that is over 22 000 ppm.

Cinnamomum verum oil was obtained from the bark of *Cinnamomum verum* J. Presl, which were collected from plants growing in distinct areas of commercial plantations in southern India. The essential oil was extracted by hydrodistillation using a modified Clevenger apparatus. The bark was pulverized and 20 g was distilled in 300 mL DH2O in a 500 mL flask for 60 min. Oil samples were stored at 4°C until bioassays.

Extracts from *Sapindus saponaria* L. and *Thymus vulgaris* L. were prepared from the seeds of *S. saponaria* or flowering plants of *T. vulgaris*, respectively, by pulverization and extracted using 100% pure methanol during 48 h at the laboratory temperature (ratio plants : methanol; 1 : 10).

Thymol (pure 99.9%) was obtained from Sigma-Aldrich, Czech Republic.

2.3. Formulations

RE: Pongam (emulsified using Tween 85—ratio Tween : Oil = 1 : 9).

REP: Pongam + thymol (emulsified using Tween 85—ratio Tween : Oil : thymol = 1 : 8 : 1).

REP3: Pongam + *Thymus vulgaris* extract (emulsified using Tween 85—ratio Tween : Oil : extract = 1 : 8 : 1).

REP4: Pongam + *Cinnamomum verum* oil (emulsified using Tween 85—ratio Tween : Oil : EO = 1 : 8 : 1).

REP5: Pongam + *Sapindus saponaria* extract (emulsified using Tween 85—ratio Tween : Oil : extract = 1 : 8 : 1).

NA: NeemAzal TS—The commercial insecticide NeemAzal-U (a.i. azadirachtin A 10 g/kg) (Trifolio-M GmbH, Lah-nau, Germany) was used for treatment.

2.4. Bioassays. To estimate insecticidal activity of the formulations in laboratory bioassays, filter paper discs impregnated with test solutions (0.25 mL/disc) were placed in the bottom and the lid of small Petri dishes (36 mm in diameter) (Corning Inc., USA); then a bean leaf treated with the same test solutions and 10 adult aphids were added. The control was treated with water. Twenty-four hours later, live and dead aphids and their offspring were counted. There were 5 replicates for each treatment. Biological efficacy, inhibition of oviposition, and mortality of subsequent nymphs were corrected by Abbott's formula [14].

The effect of the treatments on aphid larvae mortality was determined as follows. The pepper plants (*Capsicum annuum var. annuum* L.) were grown particularly in the flowerpots at 23 ± 2°C, 16 : 8 LD. The 15 aphid's first instar larvae were placed on each plant with two true leaves freshly treated with formulations or water (control). The number of live and dead aphids was counted 1, 2, 3, and 7 days after. This procedure was replicated 10 times for each formulation and the control. Efficacy was corrected by Abbott's formula [14].

Data were examined using analysis of variance (ANOVA), and means were separated using the Tukey honestly significant difference (HSD) multiple comparison test ($P < 0.05$). The LC_{50} and the 95% confidence limit of upper and lower confidence levels were calculated by using probit analysis [15].

3. Result and Discussion

At the maximum concentration (3%), almost all formulations, except REP5, resulted in 90% or above mortality of treated females (Table 1). With such a high mortality rate in tests NA, REP, REP3, and RE only a few larvae hatch and immediately died. On the other hand, after REP4 treatment, the fertility did not change significantly compared to the control, but the emergence of the next generation of individuals was not viable.

The gradual decrease in the concentration of working solutions in 2 times allowed us to determine the samples that retain their activity. In particular, even at a concentration of 0.75%, RE caused the death of more than 60% of females and inhibited the development of subsequent nymphs on 80%. After dilution to 0.375%, the efficacy (mortality of females) was 50%. The NA and REP4 somewhat inferior to RE in imagocidal activity.

When studying the influence of preparations on mortality of larvae of green peach aphid on vegetative plants, all samples showed high aphicidal activity at 3 and 1.5% (Table 2). There is a clearly expressed dynamic of aphid's death, which indicates the accumulation of toxins in the body of insects. The preparations differed in speed of onset of mortality.

Thus, the larval mortality on day 3 was over 60% in the tests with REP and REP3, while in tests with other formulations—below 50%. After dilution of the working solution to 0.75%, the activity of all samples sharply reduced, except REP (40%). The test samples are inferior to standard NeemAzal TS (NA) in the speed of appearance of the effect and retention of activity at reducing the concentration of the working solution, but nevertheless have certain larvicidal action to this insect.

The main component in our experimental formulations—*P. pinnata* oil—was not chosen by chance. So, Pongam oil treatments reduced the number of whiteflies on the chrysanthemum plants [16]. Also, the Pongamia oil caused high mortality of *Spodoptera littoralis, M. persicae,* and *Tetranychus urticae* on greenhouse plants [10].

Table 1: Activity of plant preparations to green peach aphid female and to its offspring.

Treatment	Concentration %	Biological efficacy %*	LC_{50} (CI_{95})	Inhibition oviposition %	Corrected mortality of subsequent nymphs %*	LC_{50} (CI_{95})
REP	3.0	90.3[a]	0.86 (0.72–0.96)	93.5	100.0[a]	0.59 (0.42–0.68)
	1.5	84.3[ab]		81.5	92.9[b]	
	0.75	27.2[cd]		60.9	54.4[c]	
	0.375	11.6[e]		56.5	20.8	
REP3	3.0	90.6[a]	0.93 (0.85–1.11)	89.8	100.0[a]	N.D.
	1.5	97.9[a]		100.0	—	
	0.75	28.6[d]		64.1	0.0[e]	
	0.375	16.7[de]		47.8	0.0[e]	
REP4	3.0	97.8[a]	0.95 (0.78–1.05)	28.6	100.0[a]	1.25 (1.18–1.35)
	1.5	71.5[b]		11.7	100.0[a]	
	0.75	42.8[bc]		18.2	26.6[d]	
	0.375	35.3[c]		158.6	24.1[d]	
REP5	3.0	73.5[b]	2.18 (1.98–2.25)	79.7	95.0[b]	0.89 (0.78–0.99)
	1.5	24.6[d]		60.1	66.0[c]	
	0.75	27.7[d]		75.7	42.7[d]	
	0.375	3.1[f]		48.6	0.0[e]	
RE	3.0	95.9[a]	0.36 (0.28–0.45)	97.4	100.0[a]	0.58 (0.39–0.65)
	1.5	97.8[a]		94.8	100.0[a]	
	0.75	60.4[b]		67.5	78.9[bc]	
	0.375	50.0[bc]		58.4	5.5[e]	
NA	3.0	88.6[b]	1.25 (0.96–1.38)	97.3	100.0[a]	0.72 (0.67–0.95)
	1.5	83.3[b]		78.4	91.7[b]	
	0.75	36.3[c]		53.8	63.4[c]	
	0.375	29.3[cd]		36.5	45.3[cd]	

*The values within columns with the same lowercase letter do not differ significantly (Turkey's HSD test, $P < 0.05$).

There is not enough information about insecticidal properties of *P. pinnata* oil. Considering that, it is similar to well-known neem oil by a number of properties.

Additional components of our experimental formulations have insecticidal activity *per se*.

The thymol is one of the main substances of *T. vulgaris*. The extract and oil of this plant were toxic for stored pests *Tribolium castaneum* (Herbst), *Callosobruchus maculatus,* and *Sitophilus granarius* [17, 18], *Sitotroga granarius*, *Acanthoscelides obtectus* [19], and phytophage *Trialeurodes vaporariorum* [20].

A methylene chloride extract of the *C. verum* was shown to be insecticidal to *T. castaneum* and *Sitophilus zeamais* Motsch [21]. Cinnamon oil provided >90% mortality of citrus mealybug *Planococcus citri* (Risso), but did not provide sufficient control of sweetpotato whitefly *Bemisia tabaci* (Gennadius) or green peach aphid *M. persicae* 7, 14, and 21 d after application [22].

A vast number of species showing great potential as anti-insect agents belong to *Sapindus* genus.

These plants are mostly known for being rich in saponins, which provide plant extracts with biological activities in medicine as well as in pest control [23].

Comparative evaluation of the activity of the tested formulations showed that the addition of various components to *P. pinnata* oil did not result in synergistic effects. Nevertheless, they all had aphicidal activity for *M. persicae* adults and larvae. Moreover, they possess prolonged action, exerting a negative influence on the offspring.

When preparations are recommended for pest management programs, besides the biological efficacy, the absence of side effects related to beneficial species (pollinators and insect predators) and protected plants plays a significant role.

In practice, the field treatment with 1% Pongamia oil did not have a negative influence on insect pollinators: Hymenopterans—*Apis florea*, *Apis dorsata*, and so forth, Dipterans—Muscidae, Syrphidae, and so forth, other orders—Lepidoptera, Hemiptera, and Coleoptera [24]. A high concentration of formulations we applied, (maximum 3%, at practical application, the most commonly used concentrations for formulations of plant insecticides are 0.5–1%) did not cause burns of plants (beans and peppers). In addition, the single treatment with these formulations provides significant reduction in the number of aphids during the observation period, because of the efficiency rising in time.

Table 2: Cumulating aphid mortality (mean ± SE) at different times after treatment with plant preparations.

Treatment	Concentration, %	Days, after treatment 1		2		3		7	
		Mortality %*	Biological efficacy %	Mortality, %*	Biological efficacy %	Mortality, %*	Biological efficacy %	Mortality %*	Biological efficacy %
REP	3.0	33.3 ± 8.2^{a}	27.7	61.1 ± 4.6^{a}	56.8	76.7 ± 4.8^{a}	73.1	96.6 ± 1.5^{a}	95.6
	1.5	41.1 ± 4.9^{a}	36.1	52.2 ± 6.3^{ab}	41.8	86.6 ± 3.4^{a}	79.9	97.8 ± 1.4^{a}	96.2
	0.75	13.3 ± 2.4^{c}	10.3	36.7 ± 2.8^{bc}	31.4	47.8 ± 5.0^{bc}	39.1	57.8 ± 6.8^{c}	41.9
REP3	3.0	29.9 ± 5.1^{ab}	24.1	54.4 ± 4.3^{ab}	49.4	71.1 ± 3.7^{ab}	66.7	94.4 ± 2.7^{a}	92.7
	1.5	26.7 ± 4.8^{b}	23.3	56.7 ± 5.1^{ab}	50.1	82.2 ± 7.8^{a}	77.1	96.6 ± 2.3^{a}	95.1
	0.75	2.2 ± 2.2^{d}	0	21.7 ± 3.4^{d}	15.2	40.9 ± 3.4^{c}	31.0	48.9 ± 4.1^{c}	29.6
REP4	3.0	4.4 ± 2.2^{d}	0	35.5 ± 4.7^{bc}	28.4	48.9 ± 4.7^{bc}	41.1	88.9 ± 3.3^{a}	85.5
	1.5	8.9 ± 2.8^{d}	4.7	30.0 ± 2.8^{c}	19.3	43.3 ± 2.8^{c}	27.1	79.9 ± 3.4^{b}	71.3
	0.75	3.3 ± 1.5^{d}	0	17.6 ± 3.7^{d}	10.7	37.3 ± 3.9^{c}	26.8	45.0 ± 2.9^{cd}	24.2
REP5	3.0	6.7 ± 3.4^{d}	6.7	21.1 ± 6.0^{d}	12.4	48.9 ± 9.2^{bc}	41.4	84.4 ± 4.1^{ab}	79.7
	1.5	15.6 ± 3.2^{c}	8.5	34.4 ± 3.1^{bc}	20.2	67.7 ± 8.1^{b}	12.1	95.5 ± 2.2^{a}	92.9
RE	3.0	31.1 ± 6.5^{a}	25.3	51.1 ± 7.4^{ab}	45.7	58.9 ± 7.7^{bc}	52.6	84.4 ± 5.6^{ab}	79.7
	1.5	10.0 ± 2.8^{d}	2.3	44.4 ± 7.4^{b}	32.3	62.7 ± 12.3^{b}	44.1	94.4 ± 2.1^{a}	91.2
	0.75	7.8 ± 5.0^{d}	4.6	18.4 ± 3.8^{d}	11.6	32.7 ± 2.7^{c}	21.5	49.2 ± 6.5^{c}	30.0
NA	3.0	20.0 ± 4.8^{bc}	13.3	56.7 ± 6.3^{ab}	51.9	78.9 ± 2.1^{a}	75.7	100^{a}	100
	1.5	10.0 ± 2.8^{d}	5.9	43.3 ± 4.8^{b}	34.6	84.4 ± 2.2^{a}	79.9	96.6 ± 1.5^{a}	95.1
	0.75	4.4 ± 2.2^{d}	1.1	36.7 ± 2.8^{bc}	31.4	77.8 ± 2.8^{ab}	74.1	97.8 ± 2.2^{a}	96.7
Control	for 3%	7.7 ± 2.7^{d}	—	9.9 ± 2.3^{e}	—	13.3 ± 3.8^{d}	—	23.3 ± 6.8^{d}	—
	for 1.5%	4.4 ± 3.3^{d}	—	13.3 ± 4.2^{de}	—	22.2 ± 4.4^{d}	—	30.0 ± 4.1^{d}	—
	for 0.75%	7.7 ± 4.4^{d}	—	17.8 ± 6.4^{d}	—	33.3 ± 7.3^{c}	—	36.6 ± 6.4^{d}	—

*The values within columns with the same lowercase letter do not differ significantly (Turkey's HSD test, $P < 0.05$).

Thus, the high activity of the formulations with *Pongamia pinnata* oil against the green peach aphid, absence of negative effects on pollinators, and phytotoxicity may be used as a basis for the study of their effects on complex arthropods, damaging crops in greenhouses, for inclusion in the integrated pest management program.

Conflict of Interests

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

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Research Article

A New Species of *Dikrella* Oman, 1949 (Hemiptera: Cicadellidae: Typhlocybinae) Found on *Caryocar brasiliense* Cambess. (Caryocaraceae) in Minas Gerais State, Brazil

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A new species of *Dikrella* is described and figured based on specimens from Minas Gerais, Southeastern Brazil. The new species is diagnosed by the process of pygofer and the general form of aedeagus. Adult males, females, and also nymphs were found on pequi tree, suggesting that *Dikrella caryocar* n. sp. has its full life cycle in this plant.

1. Introduction

The genus *Dikrella* Oman (Dikraneurini) includes 40 species and occurs in the United States, Mexico, Costa Rica, Cuba, Puerto Rico, Panama, Ecuador, Colombia, Bolivia, and Brazil [1–6]. In Brazil there are records of *Dikrella fumida* (Osborn) from Santa Catarina, *D. albonasa* (McAtee) from Mato Grosso do Sul, *D. aculeata* Coelho & Nessimian, *D. reticulata* Coelho & Nessimian, and *D. spinifera* Coelho & Nessimian from Minas Gerais [1, 6–9]. Studying the relationships of insects with pequi tree or souari nut (*Caryocar brasiliense* Cambess., Caryocaraceae), a new species of *Dikrella* was found [10]. According to Leite et al. [11] the species is classified as constant, occurring throughout the year, and more abundant in summer and autumn. The species is found sucking seedlings with potential to become a pest in commercial crop of *C. brasiliense*.

2. Material and Methods

The study was developed in a savannah ecosystem ("Cerrado") in Montes Claros municipality, north of Minas Gerais State, Brazil (43°55′7.3″W; 16°44′55.6″S; altitude 943 m a.s.l.). The climate and vegetation characteristics are considered in Leite et al. [10, 11].

To study the morphology of the genital apparatus it was necessary to remove the abdomen and dip it in a warmed solution of 10% KOH (modified from Oman [12]). The genitalia structures were sunk in glycerin jelly to make the illustrations [13]. The terminology was based on Young [14], except for the wings [15] and female genitalia [16, 17]. The type-specimens are deposited in the Coleção Entomológica Professor José Alfredo Pinheiro Dutra, Departamento de Zoologia, Instituto de Biologia, Universidade Federal do Rio de Janeiro (DZRJ), Rio de Janeiro, RJ, Brazil.

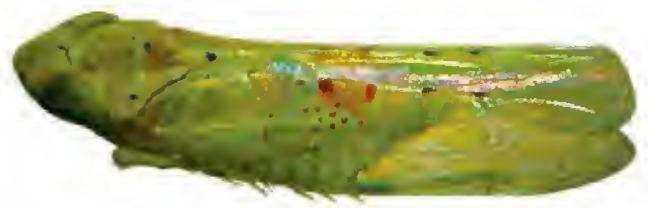

Figure 1: *Dikrella caryocar* n. sp, male: habitus (latero dorsal).

3. Results and Discussion

Dikrella caryocar n. sp. (Figures 1–3). Total length 2.7–2.9 mm. General color light green (Figure 1). Crown longer medially than next to eyes; distinct suture until just beyond half the length of crown, marked with brown at base; small smoky brown spot on each side of median line, near anterior margin. Face with small brown spot near anterior margin, between midline and inner margin of each eye. Pronotum about twice wider than long, slightly longer than median length of crown; margin of laterobasal angles not exceeding width of head; anterior margin with three irregular dark spots; lateral margin with an elongated irregular light brown spot linked to a yellowish brown spot in central region on each side of median line; a dark brown macula near each lateral angle and a black spot at middle of posterior margin. Mesonotum brown with dark brown spot on each side of median line. Scutellum pale yellow with brown longitudinal band on each side of the median line; apex with black spot. Forewing (Figure 2(a)) translucent green with big yellow spots; oval spots finely outlined with brown in apical and subapical cells; small red spots concentrated near CuP and two larger, also red, in claval area; apex of CuP and end external SmR with small brown spot. Hind wing (Figure 2(c)) with vein CuA_2 originating from point slightly more basal than MP_2. Abdominal apodeme (Figure 2(d)) long, parallel margins and apex rounded, reaching fifth segment.

Male genitalia (Figures 2(e)–2(m)): subgenital plate rather broad and triangular in ventral view (Figure 2(e)), and long in lateral view (Figure 2(f)), with apex upturned and rounded, exceeding pygofer apex; outer margin with a median dark brown lobe, from this a fold (towards inner margin of preapical region) also marked with dark brown till half width of plate; four macrosetae in outer margin, one more basal and three most apical to lobe, continued by small and robust setae till apex; microsetae present throughout surface of apical curvature. Pygofer (Figure 2(g)) elongated with five robust setae in ventroapical region; process (Figure 2(h)) elongated, dorsal in origin, with median curvature forming two branches; basal branch extending to posterior margin till ventral curve, marked by a large lateroventral tooth; apical branch free, directed posteriorly, thinner, with three small teeth on apical region, apex acute. Style (Figures 2(i) and 2(j)) elongated, preapical region curved, apex acute; preapical lobe well developed, broadly rounded, with group of five setae. Connective "Y" shape (Figure 2(k)), main stem shorter than lateral arms. Aedeagus (Figures 2(l) and 2(m)) with atrial complex developed, stem robust and laterally compressed to well-developed dorsal apodeme; tubular, slender, membranous dorsal extension (about 1/3 of stem size) coating gonoduct; pair of robust processes proceeding the main stem, with bases fused next base of membranous extension; processes apex thin and curved dorsally.

Female genitalia (Figure 3): posterior margin of seventh sternite (Figure 3(a)) with median globular prominence and each side folds forming an embossed "V" shape. Pygofer (Figure 3(b)) with nine macrosetae in ventral margin, three smaller macrosetae in posterior margin. Valvulae I (Figure 3(c)) with dorsal margin crenulated, curved towards ventral margin, apical region abruptly tapered, apex acute. Valvulae II (Figures 3(d) and 3(e)) with apical region conspicuously curved, apex narrow and rounded; right and left valvulae II asymmetrical; left valvulae (Figure 3(d)) smaller, with small rounded teeth in dorsal margin; right valvulae (Figure 3(e)) with strong teeth in dorsal margin decreasing in size towards apex. Valvulae III (Figure 3(f)) covered by short setae; three longer setae regularly spaced in ventral margin.

3.1. Studied Specimens. Holotype (male): Montes Claros, Minas Gerais State, Brazil (43°55′7.3″W; 16°44′55.6″S; altitude 943 m a.s.l.), 16/xi/2007, G.L.D. Leite leg. (DZRJ); paratypes (4 males, 10 females), same data of holotype (DZRJ).

3.2. Etymology. Karuon, Ancient Greek for "nut," "kernel"; *kará*, Ancient Greek for "head," referring to the generic epithet of pequi tree.

3.3. Comments. The color of the specimens (Figure 1) can be changed with time of conservation to a pale green or yellow. Brown spots on head and thorax may be more reddish or yellowish. No differences were found between male and female in body size. Fore wings show a variation in the studied specimens of *Dikrella caryocar* n. sp.; the base of apical cell 3 should be sessile or pedunculated (Figures 2(a) and 2(b)). This variation was found in males and females.

In male specimens the process of pygofer is robust and has a characteristic shape (Figure 2(g)) differentiated from its congeners. Aedeagus (Figures 2(l) and 2(m)) is quite unique in general shape and not similar to any other species of *Dikrella*.

In lateral view the subgenital plate (Figure 2(f)) of males of *D. caryocar* n. sp. resembles that of *D. angustella* Ruppel and DeLong and *D. venella* Ruppel and DeLong [3] by its elongated shape and rounded curved apices. The presence of a rounded lobe can differentiate the new species from

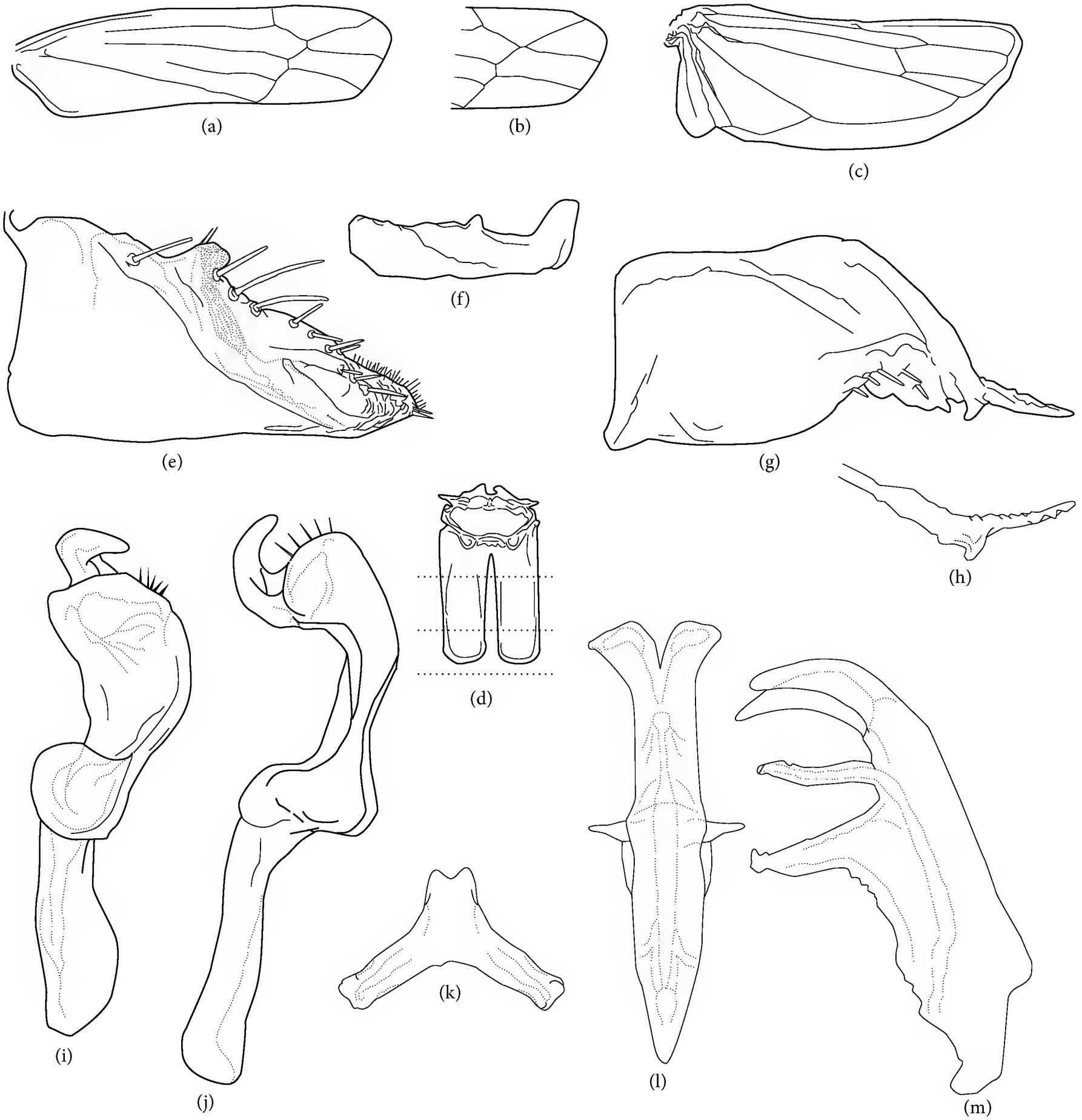

FIGURE 2: *Dikrella caryocar* n. sp., male: (a) fore wing; (b) fore wing showing a variation in base of third apical cell; (c) hind wing; (d) abdominal apodeme; (e) subgenital plate (ventrolateral); (f) subgenital plate (lateral); (g) pygofer (lateral); (h) process of pygofer (dorsoposterior); (i) style (ventral); (j) style (lateral); (k) connective (ventral); (l) aedeagus (ventroposterior); and (m) aedeagus (lateral).

D. angustella and *D. venella*. Also in subgenital plate the lobe in outer margin should superficially resembles structures present in *D. venella* Ruppel and DeLong, *D. bimaculata* Ruppel and DeLong, *D. mella* Ruppel and DeLong, and *D. nigrinota* Ruppel and DeLong [3], but in those species the prominence is in shape of one or two spines.

Adult males and females were found, as well as different nymphal instars, demonstrating that pequi tree is an ideal host for development and maintenance of the species, further suggesting that *D. caryocar* n. sp. has its full cycle in this plant.

Disclosure

The species was recorded in ZooBank under the number urn: lsid:zoobank.org:pub:0A2D5238-8998-48E7-A884-34DB31281385. The new names included in this paper are available under the International Code of Zoological Nomenclature. This work and the nomenclatural acts it contains have been registered in ZooBank. ZooBank Life Science Identifier (LSID) for this publication is urn:lsid:zoobank. org:pub: XXXXXXX. The LSID registration and any associated information can be viewed in a web browser by adding the LSID to the portal "http://zoobank.org/."

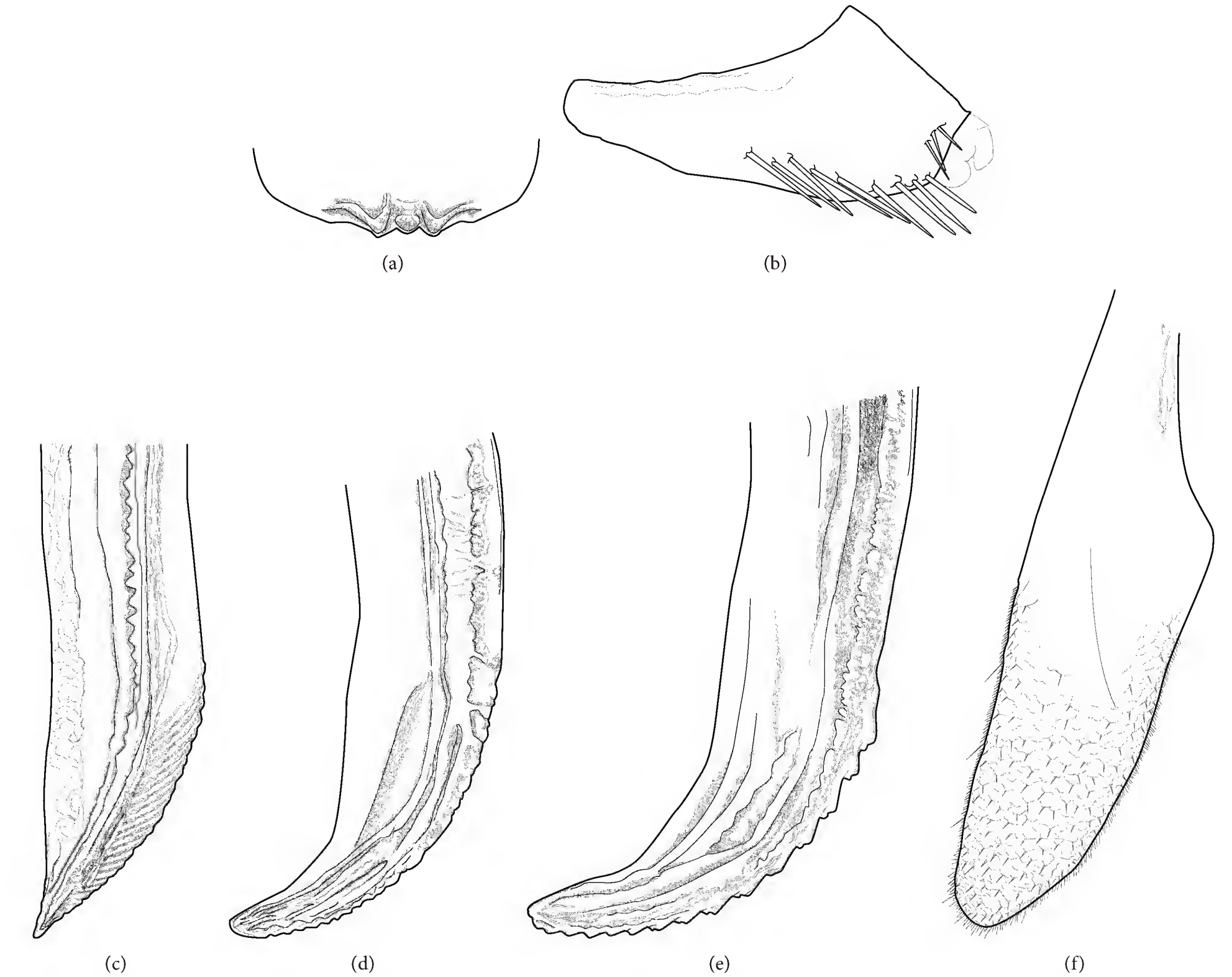

FIGURE 3: *Dikrella caryocar* n. sp. (female genitalia): (a) seventh sternite (ventral); (b) pygofer (lateral); (c) valvulae I; (d) valvulae II (left); (e) valvulae II (right); (f) valvulae III.

Conflict of Interests

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

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Research Article

Age Stage Two-Sex Life Table Reveals Sublethal Effects of Some Herbal and Chemical Insecticides on Adults of *Bemisia tabaci* (Hem.: Aleyrodidae)

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The sweetpotato whitefly, *Bemisia tabaci* (Genn.) (Hem.: Aleyrodidae), is an important pest of agriculture in subtropical and tropical areas. In this study, we used the age-stage two-sex life table to evaluate the sublethal effects of the herbal extracts taken from *Fumaria parviflora* Lam. (Fumariaceae), *Teucrium polium* L. (Lamiaceae), *Calotropis procera* (Willd.) R. Br. (Asclepiadaceae), and *Thymus vulgaris* L. (Lamiaceae) as well as the two commercial synthetic insecticides, pymetrozin and neemarin. The whiteflies were exposed to each insecticide using leaf-dip method. Analysis of life table parameters revealed significant differences ($P \leq 0.05$) in the net reproductive rate (R_0, NRR), intrinsic rate of increase (r_m), and finite rate of increase (λ) among different insecticides. The lowest values of the three population parameters, R_0, r, and λ, were observed on whiteflies treated with pymetrozin (2.455, 0.036, and 1.036), *T. polium* (2.828, 0.044, and 1.045), and neemarin (2.998, 0.046, and 1.047), respectively. Results of this study highlights the satisfactory insecticidal effects of the extract taken from *T. polium* on *B. tabaci*, which is comparable to the two commonly used synthetic insecticides.

1. Introduction

The sweetpotato whitefly, *Bemisia tabaci* (Genn.) (Hem.: Aleyrodidae) is one of the most important pests of agriculture in subtropical and tropical regions as well as in greenhouse production systems across the world [1–4]. Both adult and nymphal stages cause economic damages through a combination of direct feeding on sap phloem [5], excretion of honeydew, which serves as a substrate for growth of black sooty molds [6], and transmission of a large number of plant pathogenic viruses [7–10]. The use of chemical insecticides has long been considered as the primary strategy for control of *B. tabaci* [10, 11]. However, the rapid and frequent development of resistance against these compounds as well as the unwanted effects of synthetic pesticides on nontarget organisms and environment makes them a nonsatisfactory tool in integrated management programs of *B. tabaci*. For several decades, the plant-derived insecticides (botanicals) have been considered as potential alternatives to synthetic pesticides due to their safety to human health as well as their low detrimental effects on nontarget organisms and environment.

Plants produce a variety of chemicals that are predominantly used to defend themselves against herbivores [12]. Although, a large number of these substances have been already extracted and identified in different plant species, the availability of botanical insecticides has been limited to a few products [13]. Although, in the immediate future biopesticides may continue to be limited mainly to niche and specialty markets, there is great potential for long-term development and application in different areas of pest management science [14, 15]. A large volume of studies have been conducted to evaluate the insecticidal properties of plant-derived substances on some important pests [16–23].

Traditionally, measurement of the acute toxicity of pesticides to beneficial arthropods has relied largely on the determination of an acute median lethal dose or concentration. However, the estimated lethal dose during acute toxicity tests may only be a partial measure of the deleterious effects. In addition to direct mortality induced by pesticides, their sublethal effects on arthropod physiology and behavior must be considered for a complete analysis of their impact [24, 25]. Therefore, accurate assessment of sublethal effects is crucial to acquire knowledge on the overall insecticide efficacy in controlling insect pest populations, as well as on their selectivity towards nontarget organisms [26]. On the other hand, it has been shown that the efficacy of insecticide may be different in relation to the times they are used [27, 28]. Therefore, awareness of pest life tables and plant phenology are very important as the two consequential aspects in pest ecology. Because pest susceptibility to control agents alters between life stages, knowledge on the stage structure of a pest population is necessary for establishing the most effective pesticide application schedule [28]. Since, simulations based on the age-stage, two-sex life table explain the stage structure of a pest population at each time period, the population simulations according to this method can be efficiently used to select the best control strategy based on the stage structure [28]. In this research, we used the age-stage two-sex life table model to analyze the life table parameters of *B. tabaci*, as a new method to evaluate the sublethal effects of some botanical and synthetic insecticides. They include the extracts taken from *Fumaria parviflora*, *Teucrium polium, Calotropis procera,* and *Thymus Vulgaris* as well as the two commercially available synthetic pesticides, pymetrozin and neemarin.

2. Materials and Methods

2.1. Host Plants. We used cotton plants, *Gossypium hirsutum* var. Varamin, as the main host for mass rearing of *B. tabaci* and tomato plants, *Lycopersicum esculentum* var. Bakker brothers, as host in experiments. The cotton and tomato seeds were cultured in transplant trays at greenhouse conditions. Some stand plants were separately transferred in plastic pots (15 × 15 × 20 cm) which filled with a commercial Sterile Plant Growth Media, BAGA (Bastare Amade Giah Arganic, manufactured by Dashte Sabz Atie Co., UTSTP, Iran), while others were planted in hydroponic pots. All pots were kept in 60 × 50 × 80 cm cages covered by fine cloth mesh. Old pots were replaced with new one monthly.

2.2. Study Insects. Adult *B. tabaci*, collected from native colonies of Rafsanjan cotton fields (Kerman province, South east of Iran), was released on cotton plants in greenhouse of College of Agriculture (Vali-e-Asr University of Rafsanjan). The pupae were analyzed taxonomically and biotype A specimens were isolated and reared as original experimental source. The stock colony was reared in controlled conditions (27 ± 2°C, 50 ± 5 RH and 16 : 8 h L : D).

Young fully expanded tomato leaves were put in small plastic pots (10 cm diameter, height: 15 cm) containing distilled water. The pots were then covered by similar transparent pots to make a transparent glass cages. Adult whiteflies were released into the cages through a small pore located at the upper pot. In order to produce the same age 24 h adults, the eye-red pupa was checked daily and new born adults were collected and released into glass cages.

2.3. Pesticides. Commercial formulations of the two commonly used insecticides, pymetrozin (Chess 25% WP, Singenata Company), and azadirachtin (Neemarin EC1500), were used in this study.

2.4. Plant Extract Preparation. Four medicinal plants including fumitory, *F. parviflora* (Fumariaceae), germander, *T. polium* (Lamiaceae), swallowwort, *C. procera* (Asclepiadaceae), and thyme, *Th. Vulgaris* (Lamiaceae) were used in this study. Herbal parts of plants (leaves and flowers) were collected from natural habitats in Kerman province, southeastern Iran, during their flowering stages (May and June 2010). Plant materials of *F. parviflora*, *T. polium*,and *Th. vulgaris* were collected from Rafsanjan (30°23′46″E, 55°56′25″N), while those of *C. procera* were gathered from Jiroft (28°40′37″E, 57°43′52″N). The collected plants were identified at species level by Dr. M.A. Vakili at the Plant Taxonomy Department of the Islamic Azad University of Jiroft. Sampling was carried out from an average number of 50 plant stalks and three samples were taken from each individual plant. The plant materials were air dried for 4-5 days and coarsely powdered using an auto mixer. Twenty grams of dried materials were placed on filter paper and steeped in ethanol (90 mL) and water (210 mL) for 12 hours. These extractions were prepared according to the Soxhlet extraction method [29, 30]. Afterwards, rotation was used to reach an extract amount of one-third.

2.5. Concentration-Mortality Response. The effects of five concentrations of the six used pesticides on *B. tabaci* were assayed using the leaf-dip method. Two-leave tomato plants were dipped in pesticide dilutions for 5 s [31] and then put separately in glass cages. After drying the treated leaflets, fifteen adult *B. tabaci* were released into each cage. The cages were maintained under aforementioned controlled conditions. A solution of 3% ethanol/distilled water was used as control. The total numbers of dead adults were counted after 24 h. Adults were considered died when they were not able to move properly as a result of stimulation with a soft brush. This assay was conducted in a completely randomized design with three replicates being considered for each insecticide.

2.6. Sublethal Effects on Life History Traits. Adult whiteflies (24 hours old) were released into glass cages containing tomato stalks and allowed to oviposit for 24 h. After oviposition, adults were removed from the cages. Fifty eggs were randomly controlled every 24 h and the egg duration was recorded. After that all immature stages (nymph and pupa) growth periods were studied daily. Tomato leaflets were

dipped in the lethal concentration 25% (LC25) of each insecticide for 5 s [31]. After air drying, the plants were transferred to the glass cages. Thirty new emerged adults were randomly captured from the stock colony and released into cages. After 24 h, all adults were removed from the cages and released into new ones. The numbers of eggs laid by each female were recorded by digital microscope Dino Capture and Stereomicroscope and continued until the death of the last female. After adult emergence, the sex ratio of F1 generation was determined based on the method of Gerling [4].

2.7. Data Analysis. Probit analysis was performed to estimate the LC50 and LC25 values using Polo-Plus 2.00 software. The population parameters data were analyzed using SPSS software (version 16). One-way analysis of variance (ANOVA) and Duncan's multiple range tests were conducted to compare the effects of different insecticides on life table parameters of *B. tabaci* ($P < 0.05$). The life table parameters were analyzed using age-stage two-sex life table and female age-specific life table methods.

In age-stage two-sex life table, developmental time of all individuals, including males, females, and those dying before adult stage, as well as female daily fecundity was analyzed according to the age-stage, two-sex life table [32, 33]. Processing of raw data analysis was facilitated by a computer program; TWOSEX-MSChart [34]. No standard error was calculated for the data which was analyzed by the TWOSEX-MSChart program [34]. Various life table parameters including the age-stage specific survival rate (s_{xj}, where x = age and j = stage), the age-stage specific life expectancy (e_{xj}, where x = age and j = stage; it gave the expected time that an individual of age x and stage j will live), the age-stage specific fecundity (f_{xj}), the mean fecundity (F), which explained the contribution of an individual of age x and stage j to the future population, the age-specific survival rate (l_x), the age-specific fecundity (m_x), and the population parameters (r, the intrinsic rate of increase; λ, the finite rate of increase, $\lambda = e^r$; R_0, the net reproductive rate; and T, the mean generation time) were calculated according to Chi method [33].

The intrinsic rate of increase was appraised using iterative bisection method from the following equation using age indexed from 0 [35]:

$$\sum_{x=0}^{\infty} e^{-r(x=1)} l_x m_x = 1. \tag{1}$$

The mean generation time was explained as the time length that a population requires increasing to R_0-times of its size as the firmly fixed age distribution to obtain the stable increase rate of population. In other words, it means $e^{rT} = R_0$ or $\lambda^T = R_0$. The mean generation time (T) and the gross reproductive rate (GRR) were calculated by equations the following, respectively:

$$T = \frac{LnR_0}{r}, \qquad \text{GRR} = \sum m_x. \tag{2}$$

Table 1: The LC_{25} ratio (mg/mL), slop ± SE, lower and upper 95% confidence intervals, and χ^2 of some synthetic and botanical insecticides on *B. tabaci*.

Insecticides	Slop ± SE	LC_{25}	Limits 95%	χ^2
Pymetrozin	2.236 ± 0.357	0.089	53.99–120.47	1.936
Neemarin	1.073 ± 0.194	0.070	0.023–0.129	0.171
Th. vulgaris	1.991 ± 0.432	69.087	36.78–95.09	4.122
T. polium	2.011 ± 0.366	90.948	50.76–125.97	0.463
F. parviflora	2.936 ± 0.654	314.082	183.35–406.95	3.553
C. procera	2.100 ± 0.498	250.532	130.3 7–338.92	0.762

Chi [33] demonstrated that the relationship among the mean female fecundity (F) and the net reproductive rate (R_0) can be explained as

$$R_0 = F\left(\frac{N_f}{N}\right), \tag{3}$$

where N is the total number of individuals used at the start of the life table study and N_f is the number of the emerged female adults from these N eggs. This also means $N_f \times F = R_0 \times N$. On the other hand, the total number of offspring produced by all females is equal to the net reproductive rate multiply the cohort size. This relationship shows the accuracy in the age-stage, two-sex life table analysis [33].

In the age-specific female life table, the data were calculated according to Carey method [36]. Jackknife resampling methods were used to calculate the mean and standard error of population parameters [37].

3. Results

3.1. Concentration-Mortality Response. Log-probit regression analyses of concentration-mortality data showed that, 24 h after exposure of adult *B. tabaci* to the six studied insecticides, the LC_{25} values for different concentration of pymetrozin (106, 150, 210, 298, 420, and 593 μg/mL), neemarin, (0.060, 0.135, 0.330, 0.780, and 1.860 mg/mL), *Th. vulgaris*, (44, 58, 76, 100, 132, 173, and 228 mg/mL), *T. polium* (58, 76, 100, 132, 173, 223, 300, and 395 mg/mL), *F. parviflora* (100, 153, 234, 359, 550, and 842 mg/mL), and *C. procera* (200, 283, 400, 566, and 800 mg/mL) were 89.95 and 0.070 μg/mL and 69, 90.9, 314, and 250.5 mg/mL, respectively (Table 1).

3.2. Sublethal Effects. The age-stage survival rate (S_{xj}) (Figure 1) shows the probability that a new born pupa will survive to age x and stage j. In addition to survival, this curve also illustrates the stages difference, stages overlapping, and the variable developmental rate between individuals [33, 38]. As the figure shows that all adults emerged on the same day in all treatments, but the females survived longer than the males (Figure 1).

The l_x curve shows the probability that a newborn pupa will survive to age x. Results showed that the survival duration adult whiteflies (from birth to death) were 36, 36, 35, 33, 30, 33, and 35 days in control, pymetrozin, neemarin, *T. polium*, *C. procera*, *Th. Vulgaris,* and *F. parviflora* treatments,

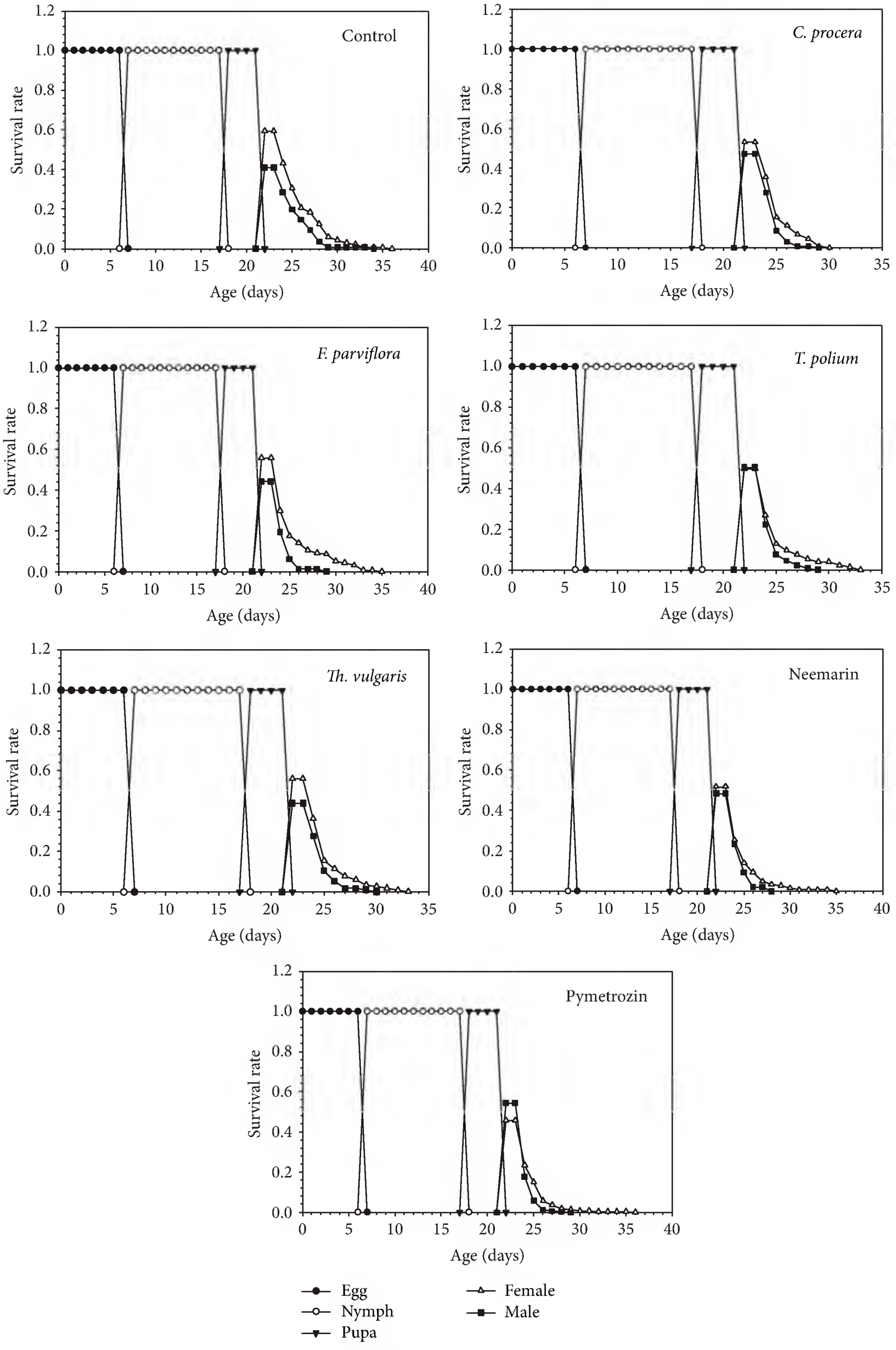

FIGURE 1: Effects of some synthetic and botanical insecticides on age-stage specific survival rate (s_{xj}) of *B. tabaci*.

respectively. The highest rate of longevity was observed in control and pymetrozin treatments (36 days), while the lowest longevity was recorded for *C. procera* extract (30 days). Our results also showed that the survival rate of females was 15, 15, 14, 12, 9, 12, and 14 days for control, pymetrozin, neemarin, *T. polium*, *C. procera*, *Th. Vulgaris*, and *F. parviflora,* respectively.

The life expectancies (e_{xj}) of adult *B. tabaci* calculated using the life duration of female and male whiteflies after the appearance of newborn adults were 4.40, 3.16, 3.21, 3.46, 3.37, 3.52, and 3.83 days for adult females and 3.94, 2.47, 2.75, 2.73, 2.85, 3.07, and 2.65 days for adult males in control, pymetrozin, neemarin, *T. polium*, *C. procera*, *Th. Vulgaris*, and *F. parviflora* treatments, respectively. The lowest life expectancy of adult females was observed in whiteflies treated by pymetrozin, neemarin, and *C. procera*, respectively.

3.3. Population Parameters. The results of statistical analysis revealed significant differences in sublethal effects of the synthetic and botanical insecticides on the net reproductive rate ($F = 8.271$; df = 6, 37; $P < 0.001$), the intrinsic rate of increase ($F = 8.619$; df = 6,37; $P < 0.001$), and the finite rate of increase ($F = 8.688$; df = 6, 37; $P < 0.001$). The average values for R_0, λ, r, and T parameters have been shown in Table 2. The highest and the lowest values of gross reproductive rate were observed in control (39.61) and *C. procera* (17.16), respectively. The lowest value of net reproductive rate was detected in pymetrozin (2.45), while the whiteflies in control showed the highest net reproductive rate. The highest amount of intrinsic rate of increase and finite rate of increase was observed in the control (0.076 and 1.079, resp.), while the lowest values were recorded for whiteflies treated by pymetrozin (0.036 and 1.036, resp.). The lowest value of mean generation time was recorded in whiteflies treated by pymetrozin (25.28) and *C. procera* (25.43). while the longest generation time was observed in those treated by *T. polium* (30.76) (Table 2).

The net reproductive rate (R_0) and the mean female fecundity (F) of control, pymetrozin, neemarin, *T. polium*, *C. procera*, *Th. Vulgaris*, and *F. parviflora* were 7.32, 2.45, 2.99, 2.82, 3.18, 3.67, and 4.91 offspring and 14.15, 5.38, 5.97, 5.58, 6.68, 6.45, and 9.07 egg/adults, respectively.

3.4. Comparison of Age-Stage Two-Sex Life Table and the Female Age-Specific Life Table. To make a comparison between the efficiency of two commonly used models, the population parameters of whiteflies treated by all insecticides were simultaneously calculated using both age-specific female life table and age-stage two-sex life table (Table 3). Statistical analyses revealed no significant difference among parameters calculated by these models.

4. Discussion

In the present study, we conducted some bioassays to assess the sublethal effects of pymetrozin and neemarin as well as the extracts taken from *F. parviflora*, *T. polium*, *C. procera*, and *Th. vulgaris* on demographical parameters of *B. tabaci*. Meanwhile, we compared the age-stage two-sex life table and the female age-specific life table to clarify the differences between the two methods. The life-table studies under different environmental conditions and on different host plants have been proposed to be a relatively time-consuming process; thus the use of life tables in pest management programs seems not to be appropriate. However, the life table studies provide us with some basic knowledge about the biological properties of studied pests, without them development of an appropriate control strategy is impractical [38].

The traditional age-specific life tables [39–41] concentrate only on the survival and the fecundity of the female population, thus ignoring the male population, the stage differences, and stage overlapping leading to a miscalculation in the survival and fecundity curves [33, 42]. To overcome these problems, the age-stage two-sex life table has been developed by Chi and Liu [32] and Chi [33]. The age-stage two-sex life table has been widely used to study the population dynamics of insect [38, 43–45]. As far as we know, this study is the first one that uses the life table parameters as a suitable index for evaluation of sublethal effects of insecticides on *B. tabaci*.

Our results showed that the female whiteflies survived longer than the males and the lowest survival rate of females was observed in *T. Polium* followed by neemarin. In the usual condition without application of pesticides, female whiteflies have been shown to survive longer than the males [43].

In our study, the overlapping in curves of S_{xj} shows the potential of the age-stage two-sex life table in displaying the stage dissociation of *B. tabaci* due to variable developmental rate between individuals. Similarly, stage differentiation can be perceived in curves of e_{xj}. In addition to stage overlapping, a correct relationship between R_0 and F can be received. In our research, the total number of offspring produced by all females was nearly equal to the net reproductive rate multiplied by the cohort size and the minor difference was attributed to rounding-off. This equality shows the accuracy of the age-stage two-sex life table analysis. These results in all treatments were consistent with the relationship obtained by Chi [33], and Yang and Chi [43].

According to our results, the survival of a population can be predicted at each condition. Our results are in line with those of Chi and Su [42], Yang and Chi [43], and Hu et al. [38] who showed the variable developmental rate and overlapping among different stages using age-stage two-sex life table. Results of our current study clearly showed that the susceptibility to pesticides and botanical compounds varied significantly among different developmental stages of *B. tabaci*. These findings are in accordance with those results of Liu and Stansly [46] on *B. argentifolii*.

Yang and Chi [43] proved that $R_0 \leq F$ meaning that the net reproductive rate is lower than the mean female fecundity. If there was preadult mortality, R_0 is expected to be lower than F, a condition that was shown by our results as well. In contrast to our results, Liu and Stansly [47] reported that the net reproductive rate was higher than the mean fecundity (i.e., $R_0 > F$). This repugnance may be related to the methods they used for calculation of R_0 and F.

The results of comparison between the age-stage two-sex life table and the traditional age specific life table indicated that consideration of male whiteflies in calculation of life table

Table 2: Comparison of life table parameters of *B. tabaci* calculated by two different methods: the age-specific female life table and the age-stage two-sex life table; different letters show significant differences at 0.05 level.

Treatments	The intrinsic rate of increase (r)		The finite rate of increase (λ)		The net reproductive rate (R_0)		The mean generation time (T)	
	Female life table	Two-sex life table	Female life table	Two-sex life table	Female life table	Two-sex life table	Female life table	Two-sex life table
Control	0.080 ± 0.007	0.076 ± 0.006	1.08 ± 0.007	1.079 ± 0.006	8.85 ± 1.89	7.23 ± 1.13	26.33 ± 0.22	26.23 ± 0.23
Pymetrozin	0.045 ± 0.017	0.036 ± 0.004	1.04 ± 0.018	1.036 ± 0.004	3.09 ± 0.14	2.45 ± 0.29	33.30 ± 8.28	25.28 ± 0.30
Neemarin	0.041 ± 0.009	0.046 ± 0.004	1.04 ± 0.010	1.047 ± 0.004	2.78 ± 0.07	2.99 ± 0.30	28.18 ± 7.08	25.64 ± 0.53
T. polium	0.048 ± 0.005	0.044 ± 0.005	1.06 ± 0.010	1.045 ± 0.006	4.46 ± 0.17	2.82 ± 0.58	31.78 ± 6.14	30.76 ± 4.38
C. procera	0.041 ± 0.009	0.049 ± 0.002	1.04 ± 0.009	1.050 ± 0.002	2.67 ± 0.06	3.18 ± 0.24	26.27 ± 6.59	25.43 ± 0.28
Th. vulgaris	0.064 ± 0.011	0.058 ± 0.004	1.06 ± 0.012	1.059 ± 0.004	4.95 ± 0.24	3.67 ± 0.33	31.62 ± 5.99	26.63 ± 0.73
F. parviflora	0.069 ± 0.006	0.064 ± 0.003	1.07 ± 0.006	1.065 ± 0.003	5.70 ± 0.14	4.91 ± 0.55	27.07 ± 2.88	26.78 ± 0.41

Table 3: Results of t-test analyses showing differences between the age-specific female life table and the age-stage two-sex life table in estimation of life table parameters of *B. tabaci*.

Treatments	The intrinsic rate of increase (r)		The finite rate of increase (λ)		The net reproductive rate (R_0)		The mean generation time (T)	
	P	t	P	t	P	t	P	t
Control	0.661	0.449	0.655	0.457	0.492	0.707	0.774	0.293
Pymetrozin	0.670	0.434	0.633	0.486	0.048	2.126	0.317	1.040
Neemarin	0.773	−0.293	0.807	−0.249	0.353	−0.958	0.798	0.262
T. polium	0.679	0.425	0.266	1.162	0.011	3.019	0.905	0.122
C. procera	0.556	−0.602	0.590	−0.550	0.016	−2.700	0.919	0.103
Th. vulgaris	0.643	0.475	0.604	0.533	0.008	3.183	0.491	0.710
F. parviflora	0.526	0.652	0.499	0.696	0.124	1.644	0.936	0.082

indices and variable developmental rates had little effects on population parameters of *B. tabaci*. The suitability of age stage two-sex life table for calculation of population parameters has been also approved by several previous studies [44].

In recent decades, with the increasing knowledge on detrimental effects of chemical pesticides on human, environment, and nontarget organisms (e.g., development of resistance by key pests, environmental pollution, human health dangers, and pest resurgence), the use of environmentally friendly compounds with the least side effects on nontarget organisms and environment have received relatively great attention. Among these compounds, the plant-derived insecticides have been traditionally considered as efficient candidate alternative to synthetic insecticides [15, 48]. A large numbers of studies have clarified that the extracts taken from the four plant species, *T. polium*, *F. parviflora*, *Th. Vulgaris*, and *C. procera*, have insecticidal properties [16–22, 49]. Our current study, however, showed that in addition to the direct mortality caused by these compounds they may also impose some sublethal effects on target pests. These effects included decreased growth, fecundity, and survival rate, increased development time, and probably increased susceptibility to natural enemies. In our study, the lowest values of r_m were observed in whiteflies treated by pymetrozin, *T. polium*, and *C. procera*. Therefore, the two mentioned botanical insecticides seem to be suitable for integrated management programs of *B. tabaci*. Altogether, results of the current study showed that some biological characteristics of the cotton whiteflies are affected at sublethal concentrations of the studied insecticides. These effects are easily distinguishable using the age stage two-sex life table method used in this study. However, before decision making for establishment of a control strategy using these insecticides, their effects on nontarget organisms, especially natural enemies, should be also evaluated.

Conflict of Interests

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

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Research Article

A Survey of Bedbug (*Cimex lectularius*) Infestation in Some Homes and Hostels in Gboko, Benue State, Nigeria

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A Survey of bed bug infestation in some homes and hostels, in Gboko, Benue State, Nigeria, was conducted from January to April, 2011. Bed frames, bunks, mattresses, pillows, chairs, and clothes were inspected. A total of 2,642 bed bugs were collected. 73.3% were from hostels while 26.7% were from homes. There was a significant difference between in the number of homes infested and those not infested ($\chi^2 = 61.44$, df $= 4$, $P < 0.05$). Nymphs were the most populated, with 292 (41.4%), followed by males 223 (31.6%), and females 190 (27.0%). There was no significant difference in the number of infested hostels and those not infested ($\chi^2 = 0.8$, df $= 4$, $P < 0.05$). The nymphs being the most populated with 901 (64.1%), followed by males 538 (36.1%), and then females 496 (35.3%). The greater number of infestation recorded in the hostels was as a result of poor hygiene, lack of adequate knowledge of the best control practices and the high population density. In homes, lack of the awareness of the resurgence of the emerging pest and lack of proper health education is responsible for the high infestation. Proactive approach should be taken towards public health education against bed bug infestation. Government and NGOs should take critical steps in preventing spread and stigma.

1. Introduction

Bedbugs are small parasitic insects of the family Cimicidae (most commonly *Cimex lectularius*). Two species are associated with humans, *Cimex lectularius and Cimex hemipterus,* which are cosmopolitan or found in tropical and subtropical regions, respectively [1]. Bedbugs are blood sucking ectoparasites that infest human habitations and usually feed during the night when the host is sleeping [2]. Under optimal conditions, the adult bedbug feeds once a week. The major attractants appear to be human body temperature and carbon dioxide production and also by certain chemicals [2].

Bedbug infestation associated problems include lack of sleep and psychological and social distress from society's stigma concerning pests [2]. Although bedbugs have not been linked to disease transmission, they have been shown to harbor the causative organisms of plague, relapsing fever tularemia, Q fever, and *Wolbachia*. Symptoms from their bites include severe irritation, itching, inflammation, and swelling of the skin [3].

Special nocturnal search is often required as the definitive diagnosis depends upon collection and identification of bedbugs [1].

In the 1980's bedbugs were considered relatively uncommon in many developed countries, such as the UK probably due to better building practices, better education, and emphasis on wide use of insecticides [4]. In developing countries, bedbug infestation is at a high level [5]. Recent observations suggest that urban settings have experienced increased infestation over the past 10 years [6–8]. There is also recent evidence for insecticide resistance in bed bugs in addition to effective application techniques [9–13]. An increasing poor attitude towards housekeeping and poor hygiene is responsible for a high infestation in Makurdi and Otukpo [14].

This survey was carried out to determine the status of bed bug infestation in Gboko, with a view to investigate control measures adopted by residents against the pest; as well as their knowledge on the hazards associated with harboring bedbugs.

Table 1: Sex and growth stage of bedbugs (*Cimex lectularius*) collected from homes in Gboko.

Location Gboko	Number of homes inspected	Number of homes infested (%)	Males (%)	Females (%)	Nymphs (%)	Total number of bedbugs (%)	Eggs (%)
Central	107	12 (11.2)	42 (31.8)	30 (22.7)	60 (45.5)	132 (18.7)	313 (16.6)
East	101	7 (6.9)	2 (24.2)	18 (19.8)	51 (56.0)	91 (12.9)	273 (14.5)
West	118	20 (16.9)	50 (27.5)	60 (33.0)	72 (40.0)	182 (25.8)	401 (21.3)
North	132	38 (28.8)	73 (33.0)	67 (30.3)	81 (36.7)	221 (31.3)	701 (37.2)
South	142	9 (6.3)	36 (45.6)	15 (19.0)	28 (35.4)	79 (11.2)	196 (10.4)
Total	600	86	223	190	292	705	1884 (100)

($\chi^2_{\text{calculated}} = 61.44$, df = 4, $\chi^2_{\text{Tabulated}} = 4.278$ at 95% level of significance).

Table 2: Sex and growth stage of bedbugs (*Cimex lectularius*) collected from Hostels in Gboko.

Location Gboko	Number of Hostels Inspected	Number of homes Infested (%)	Males (%)	Females (%)	Nymphs (%)	Total number of bedbugs (%)	Eggs (%)
Central	2	1 (50.0)	59 (19.0)	48 (15.5)	203 (65.5)	310 (16.0)	307 (21.8)
East	2	1 (50.0)	78 (25.0)	81 (26.0)	153 (49.0)	312 (16.1)	178 (12.7)
West	1	1 (100.0)	111 (27.8)	107 (26.8)	182 (25.5)	400 (20.7)	341 (24.3)
North	2	2 (100.0)	198 (32.9)	201 (33.4)	201 (33.4)	602 (31.1)	301 (21.4)
South	2	1 (50.0)	92 (29.4)	59 (18.4)	162 (51.8)	313 (16.2)	279 (19.8)
Total	9	6	538	496	901	1937	1406 (100)

($\chi^2_{\text{calculated}} = 0.8$, df = 4, $\chi^2_{\text{Tabulated}} = 4.278$ at 95% level of significance).

2. Materials and Method

The survey was conducted in Gboko, Local Government area of Benue state, Nigeria, from January to April, 2011. Gboko is located on 7°19′30″N 9°0′18″E and lies in the Savanna region of North-Central Nigeria, with a temperature range from 29°C to 33°C. It has a population of over 500,000 people who are predominantly farmers and civil servants. They speak Tiv and English language. Benue State covers an area of about 34,059 km^2 with a population of over 4.2 million people.

Written informed consent was obtained from the Gboko Local Government Health Authority and verbal consent from heads of household heads and owners of hostels. Randomly selected homes and hostels were visited and bed frames, mattresses, bamboo beds, carpets, mosquito nets, benches, walls, cushioned chairs, pillows, and bed sheets were thoroughly inspected for bedbugs infestation. Collecting bottles, paint-like brushes, and 70% alcohol were the materials used. Where bedbugs were seen, they were brushed into the collecting bottles. The material from which the bedbug was collected was noted and the number of bedbugs collected per material was noted. Residents were interviewed on the control measures adopted against bedbugs. The specimens transferred to the General Zoology Laboratory of the Benue State University for identification according to published keys by Pratt and Smith [15].

3. Result

Out of the 600 homes and 9 hostels surveyed, 86 (14.3%) homes and 6 (66.7%) hostels were infested with bedbugs. A total of 705 bedbugs were collected from homes, comprising 223 (31.6%) males, 190 (27.0%) females, and 292 (41.4%) nymphs. 1884 eggs were also collected. A total of 1937 beg bugs were collected from hostels. This comprises 538 (27.8%) males, 496 (25.6%) females, and 901 (46.5%) nymphs.

A total of 2,642 bedbugs were collected from all the hostels and homes, comprising 761 (28.8%) males and 686 (26.0%) females and 1,193 (45.2%) nymphs alongside 3290 eggs. 73.3% of the total collected bedbugs were from hostels while 26.7% of the total bedbugs were from residential homes. Gboko North had the highest number of infestation rate of 221 (31.3%) in residential homes and 602 (31.1%) in hostels, while Gboko South had the least infestation rate of 79 (11.2%) in residential homes and 313 (16.2%) in hostel (Tables 1 and 2). There was a significant difference between the number of infested and uninfested homes. In the residential homes ($\chi^2_{\text{calculated}} = 61.44$, df = 4, $\chi^2_{\text{Tabulated}} = 4.278$ at 95% level of significance). In the hostels there was no significant difference between the number of infested and uninfested hostels ($\chi^2_{\text{calculated}} = 0.8$, df = 4, $\chi^2_{\text{Tabulated}} = 4.278$ at 95% level of significance).

A total of 3,602 items were inspected, out of which 760 (21.1%) items were infested. Bed frames had highest infestation; out of 700 inspected bed frames, 314 (44.9%) were infested with a total number of 1,356 bedbugs. 11 bamboo beds were inspected and only 9 (81.8%) were infested with a total number 28 bedbugs (Table 3).

The survey of different control measures used at different sites revealed a total of 8 different control measures. A total of 600 homes use one or more of the measures. Those who use *Nuvan,* dichlorvos (2,2-dichlorovinyl dimethyl phosphate) had the highest frequency of 196 (164.0%) while those who use *Omo* (detergent) and soap had the least frequency of 27 (22.2%) (Table 4). Insect powder is mostly used outdoors, and furniture assumed to be infested is taken away from the main building and the powder is applied to harborages. Snipper and *Nuvan* are applied inside houses mostly at night. Similarly, syringes are used to spray the insecticide in corners of the house where the pest is likely to be hiding. The same insecticide is also used with the aim at controlling other insects in the houses visited.

Table 3: Distribution of bedbugs in different harborages in homes and hostels in Gboko.

Harborages	Total number of harborages	Number of harborages with bedbugs (%)	Total Number of bedbug
Bed frames	700	314 (44.9)	1,356
Mattresses	905	192 (21.2)	603
Pillows	800	93 (11.6)	262
Carpets	26	13 (50.0)	62
Walls	600	86 (14.3)	157
Wooden chairs	107	14 (13.1)	53
Wooden benches	36	9 (25.0)	24
Bamboo beds	11	9 (81.8)	28
Executive chairs	217	13 (6.0)	36
Other Furniture	200	17 (8.5)	61
Total	3,602	760	2,642

4. Discussion

This survey recorded a high rate of infestation of bedbug. This agrees with the other findings in Australia [7, 8], in Canada [16], and in Benue State, Nigeria [14].

This is as a result of the increase in the movement of people (including students from holidays and prisoners) from where infestation is very common via infested luggage, clothing, and other personal belongings and the increasing movement of bedding, furniture, and other materials by foot, car, and train. Recent increase in incidence of bedbug infestations in Canada, Italy, UK, USA and many other developed western countries has been linked to increase in international travel and the reduction in the use of insecticides to control cockroaches and ants (TerPoorten and Prose, 2005). 85.8% of the very recent cases of bedbug infestations recorded in Central Italy were recorded in apartment that housed immigrants and tourists from Mediterranean countries [17].

The data collected indicated high percentage of males than females and yielded greater percentage of nymphs. This agrees with a finding in Gbajimba, a settlement in Benue, where records show a greater percentage of males than females [14]. The greater percentage of nymphs recorded is an indication of the possibility of an increase in infestation where the right control measures are not employed. The greatest percentage of eggs points to a possible persistence of infestation by adult females. This percentage may be due to treatments applied which might have killed some adults without affecting the eggs.

The infestations were highest in bed frames; these include single or double bunk (iron beds) frames and wooden bed frames. This is as a result of conducive hiding places the harborage provide and their close proximity to their feeding or host location. This however disagrees with Doggett's findings, who indicated that iron bed harbor less bedbugs in his survey [7].

The percentage recorded in hostels is probably due to poor or bad housekeeping and poor hygiene which is very common in Gboko. The World Health Organization suggests that poor housekeeping encourages bedbug breeding [18].

A review of the control measures (treatment) used at different sites indicated a high percentage on the use of *Nuvan* (2,2-dichloroethenyl phosphate) as it was the chemical method that was easily and readily available and affordable by most residents. Lack of technical knowledge of the control measures, knowledge of their hiding places, knowledge of resistance of eggs to spraying insecticides, and wrong use of the insecticides may have contributed to the high rate of infestation [4, 9–11, 13].

Infestation of bedbugs in homes and students hostels is epidemiologically significant. The nuisance they cause may result into sleeplessness, anxiety, and insomnia which could affect concentration in school and work.

The high infestation is likely to arise from greater dispersal of bed bugs, control strategies that are not fully effective, partial treatments that disrupt but do not eradicate infestation are used which fragment and spread bed bug colonies rather than disintegrating them. The research indicates that most people have little knowledge about bed bug control measures.

Conclusively high rate of bedbug infestation recorded in some homes and hostels is no doubt a reflection of poor hygiene, sanitation, and lack of adequate knowledge concerning the pest.

It is important that proactive approach should be taken on public education and awareness against bedbug. Critical steps in preventing bedbug spread and stigma should be taken up by the government and nongovernmental organizations. Training is an important component that should be offered and tailored to stakeholders, like property owners, pest management professionals, and staff working in the residential setting.

Table 4: Treatment methods employed by residents in homes and hostels in Gboko.

Location Gboko	Total number of homes	Number of Homes infested (%)	Insect powder	Net spray insecticide (%)	*Nuvan* (%)	Kerosene (%)	Exposure under sun (%)	Burning (%)	Soap and *Omo* (detergent)	Abandoning
Central	107	12 (11.2)	28 (26.2)	6 (6.5)	37 (34.6)	9 (8.4)	11 (10.3)	5 (4.7)	5 (4.7)	6 (5.6)
East	101	7 (6.9)	23 (22.8)	4 (4.0)	34 (33.7)	14 (13.9)	11 (10.9)	7 (6.9)	3 (3.0)	5 (5.0)
West	118	20 (16.9)	31 (26.3)	5 (4.2)	37 (31.4)	13 (11.0)	13 (11.0)	7 (5.9)	6 (5.1)	6 (5.1)
North	132	38 (28.8)	33 (25.0)	5 (3.8)	44 (33.3)	16 (12.1)	16 (12.1)	9 (9.0)	5 (3.8)	4 (3.0)
South	142	9 (6.3)	32 (22.5)	8 (5.6)	44 (31.0)	19 (13.4)	14 (9.9)	10 (7.0)	8 (5.6)	7 (4.9)
Total	600	86 (70.1)	147 (118.3)	28 (23.2)	196 (184.0)	71 (58.8)	65 (54.2)	38 (33.5)	27 (22.2)	28 (23.6)

Conflict of Interests

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

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Research Article

Nothochrysinae (Neuroptera: Chrysopidae): New Larval Description and Generic Synonymy, with a Consideration of Generic Relationships

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Semaphorant B of *Kimochrysa africana* (Kimmins) expresses all of the larval synapomorphies that characterize the subfamily Nothochrysinae. Except for its head markings, the larva appears identical to that of *Hypochrysa elegans* (Burmeister). Based on consideration of both larval and adult similarities, *Kimochrysa* (Tjeder) is designated to be a subjective synonym of *Hypochrysa* Hagen (*New Synonymy*). The morphological basis for a previously proposed generic subdivision of Nothochrysinae is evaluated; the results indicate that the subfamily can be organized into two generic groupings each with distinct suites of shared adult characters. As yet, apomorphic support is not forthcoming from adult characters, and, unfortunately, larvae are known from only a few genera in the subfamily.

1. Introduction

Chrysopid taxonomists generally agree that the subfamily Nothochrysinae is an archaic, probably monophyletic grouping [1–7]. Nevertheless, synapomorphic adult features for the subfamily have been elusive, and proposals regarding the monophyly of the subfamily largely rested on the retention of presumed plesiomorphic character states, mostly in wing venation.

In contrast, recent investigations have identified several larval features (listed in a later section) that may lend apomorphic support for the monophyly of Nothochrysinae [7–10]. These studies also indicate that larvae within the subfamily express a range of variation in their overall body form—from naked to light debris carrying.

To date, larvae are described for only three of the nine genera of Nothochrysinae; thus, the taxonomic breadth of the recently recognized morphological support for the subfamily is limited, and the range and the phylogenetic value of larval variation among genera remain unknown. In the mid 1800s, Brauer [11] provided the first description of a larva from the Nothochrysinae; his article illustrated and described the monotypic European *Hypochrysa* Hagen (Semaphorant B—second or third instar, as *Hypochrysa nobilis* Heyd.). More recently, modern descriptions of the first and third instars of this species appeared [8, as *Hypochrysa elegans* Esben-Petersen]. All instars of the lone North American and the two European species of *Nothochrysa* McLachlan are described (see [7, 8, 12]). Finally, the first instars of *Dictyochrysa fulva* Esben-Petersen were described and compared with those of *Nothochrysa* [13].

In 2010, Duelli et al. [14] published images and biological notes on the larvae of *Kimochrysa africana* (Kimmins). These authors made the specimens available for morphological study and description. Here, I describe and compare the *K. africana* larvae with those known from other Nothochrysinae. As a result of the comparison, the genus *Kimochrysa* Tjeder is shown to be synonymous with *Hypochrysa* and questions arise concerning the currently held generic groupings of Nothochrysinae. These questions are addressed.

2. Materials and Methods

The specimens were collected in the Republic of South Africa, Hoek-se-Berg Pass nr. Bushmans Kloof, 32°07′04.9″ S, 19°10′29.7″ E, 650 m, 2-X-2004 (see [14]). Unfortunately an early shipment of larvae was lost in the mail; the second shipment contained second instars ($n = 3$) preserved in alcohol. Upon arrival, the specimens were photographed and the external gross features were described. One of the specimens was cleared in KOH and transferred to glycerine for examination of fine structures and setation. Two specimens are now returned to P. Duelli, Swiss Federal Research Institute WSL, Birmensdorf/Zurich, Switzerland; the cleared specimen is in the Tauber Research Collection.

Morphological terminology and chaetotaxy followed the usage of Rousset [15], Tsukaguchi [16], Tauber et al. [17], and Monserrat and Díaz-Aranda [8]. The larval stage of the Chrysopidae typically includes three instars, the first of which (Semaphorant A) is markedly different from the latter two (Semaphorant B), which resemble each other very closely except for size and small differences in chaetotaxy. Thus, it is appropriate to compare the second instars described here with third instars of other species.

3. Second Instar *Kimochrysa africana*

3.1. Diagnosis. The *K. africana* larvae express the three features previously identified as potential synapomorphies for Nothochrysinae [7], including (i) antenna: terminal segment is short (~ten times shorter than remainder of antenna), (ii) antenna: terminus has a group of small apical setae, not an elongate seta, (iii) labial palpus: terminal segment has more than three lateral sensilla.

As Duelli et al. [14] noted, the *K. africana* larvae closely resemble those of the nothochrysine *H. elegans* in their elongated, naked bodies and their bright green coloration. They also share the following morphological features: (i) head: primary setae are blunt, (ii) thorax: lateral tubercles (LTs) are absent, (iii) abdomen: LTs are absent, (iv) abdomen: laterodorsal tubercles are absent or very small and with only one seta, and (v) types of setae: thoracic notal setae are short, blunt to slightly clavate, and abdominal (A1–A6) submedian setae are short, blunt to clavate, without hooks.

The most notable difference between the larvae of the two species is that *K. africana* lacks the dark, elongated median head marking of *H. elegans*.

3.2. Description

3.2.1. Body (Figures 1(a) and 1(b)). Length ~5.9-6.9 mm (measured in lateral view through spiracles), depth ~0.85–1.1 mm (thickest section of abdomen). Bright green coloration of living specimens [14] faded in preserved specimens; dorsal surface largely cream-colored to tan, with pronotal sclerites brown, with pair of broad, reddish brown, vertical bands along lateral margins, extending from cervix to tip of A9. All setae short, smooth, pale, and of two types: "blunt/clavate" with blunt or slightly enlarged tip, usually erect and straight (primary cranial setae, dorsal thoracic, and abdominal setae (submedian setae, SMS)); "simple" with acute tip, usually erect and slightly curved (setae on cephalic appendages, some small, usually secondary, cranial setae, some very small setae on thoracic notum, setae on legs, posterior part of A9, A10, and ventral setae).

3.2.2. Head (Figures 1(d)–1(f), 2(a) and 2(b)). Dorsum cream-colored, with light brown to brown markings as in Figures 1(d) (dorsal), 1(e) (ventral), and 1(f) (lateral); eyes with stemmata clear, surrounding integument dark brown; cranium tapered posteriorly and roughly triangular, with rounded posterior (dorsal view); width (across eyes) ~0.60–0.64 mm, length (dorsum) ~0.52–0.54 mm, and depth (midregion to top of eye) ~0.13 mm; base fully exposed. Anterior margin of labrum protruding slightly, straight anteriorly, and rounded laterally. All dorsal primary cephalic setae present (Figures 2(a) and 2(b)), blunt to slightly knobbed apically; two Vx setae present; labrum with two or three pairs of setae (one mesally and two laterally); dorsum with several secondary setae: one long, indistinguishable from posterior primary setae, others very short, mesal to S6. Ventral primary setae (S9, S10) present; S8 absent.

3.2.3. Cephalic Appendages (Figures 1(d)–1(f), 2(a) and 2(b)). Mandible long, thin, with length ~1.0-1.1 mm, width ~ 0.10 mm; ratio of mandible length to head width 1.54–1.63; ratio of mandible length to head length (dorsal) 1.90–1.00. Mandible slightly upturned distally, with single acute basolateral seta; terminus sharp, with six teeth. Antennal length 0.90–0.97 mm, ~1.7-1.8x length of cranium; width ~0.03-0.04 mm (at widest part of pedicel); scape with straight sides, two pairs of distal setae (one lateral, other mesal); pedicel long, about 17–19x length of flagellum, slightly broader than the base of flagellum; flagellum short, stubby, basal flagellomere with or without mesal seta, terminus with short basolateral seta, several very short setae. Labial palp long, slender, ~0.75x length of mandible; basal segment with one short, dorsal seta basally, three setae distally (two mesal, one lateral); middle segment long, with long, undivided basal subsegment bearing ~ five setae, five shorter mesal subsegments, with one seta mesally, elongated terminal subsegment, with two long setae distally; terminal segment ~one-third length of middle segment, slightly tapered distally, terminus with several very small setae; palpiger erect, with relatively straight sides, with one mesal seta, one lateral seta; mentum with smooth plate mesal to stipes, lateral to palpiger, with three pairs of long setae; cardo and stipes elongate, narrow, longitudinally arranged; cardo behind stipes. *Cervix* expanded laterally, ventrally, withdrawn from cranium dorsally; with pair of setae dorsolaterally, two pairs laterally, two pairs ventrally.

3.2.4. Thorax (Figures 1(c) and 3(a)). Lateral tubercles absent; dorsum with scattered, short, blunt to slightly clavate setae; primary setae unidentified (except as noted below). *Legs* (Figure 1(g)) cream-colored; coxae with diffuse, light brown marks anterolaterally; trochanter, femur with dark brown, longitudinal stripes anterodorsally, posterodorsally, with dark vertical stripe at tip of segment; tibia with dark brown vertical

(a)

(b)

(c)

(d)

(e)

(f)

(g)

FIGURE 1: *Hypochrysa africana* Kimmins, second instar (Hoek-se-Berg Pass, South Africa). (a) Body, dorsal. (b) Body, lateral. (c) Thorax, dorsal. (d) Head, dorsal. (e) Head, ventral. (f) Head, lateral. (g) Legs, dorsal.

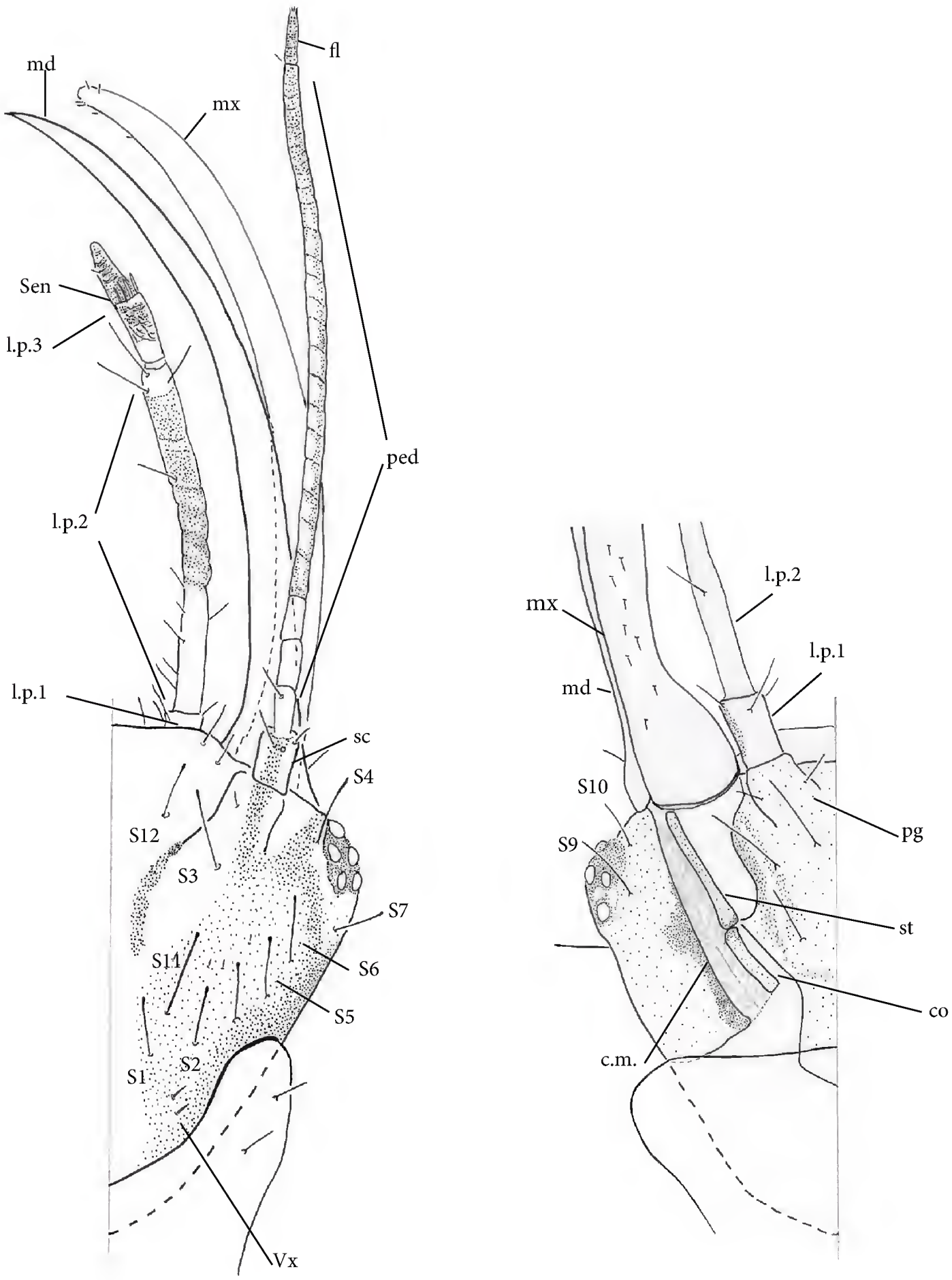

FIGURE 2: Head of *Hypochrysa africana* Kimmins, second instar. (a) Dorsal. (b) Ventral. Scale applies to both (a) and (b). Abbreviations: co, cardo; c.m., cranial margin; fl, flagellum; l.p.x, labial palpus, number of segment; md, mandible; mx, maxilla; ped, pedicel; pg, palpiger; sc, scape; sen, sensilla; stp, stipes; Sx, primary seta, number; Vx, Vx setae.

stripe basally, short, diffuse, light brown, longitudinal marks anterobasally, posterobasally; tarsus, claw, empodium dark brown.

Prothorax (T1) with two well delineated subsegments separated by transverse depression. Sc1 brown, elongated, extending almost to the full length of anterior subsegment, narrow anteriorly, posteriorly, broad mesally, embedded within lateral stripe, delineated laterally by curved, cream-colored strip. Sc2 elongated, narrow, extending posteriorly to margin of anterior subsegment.

Mesothorax (T2) consisting of three well delineated subsegments separated by distinct depressions. Anterior

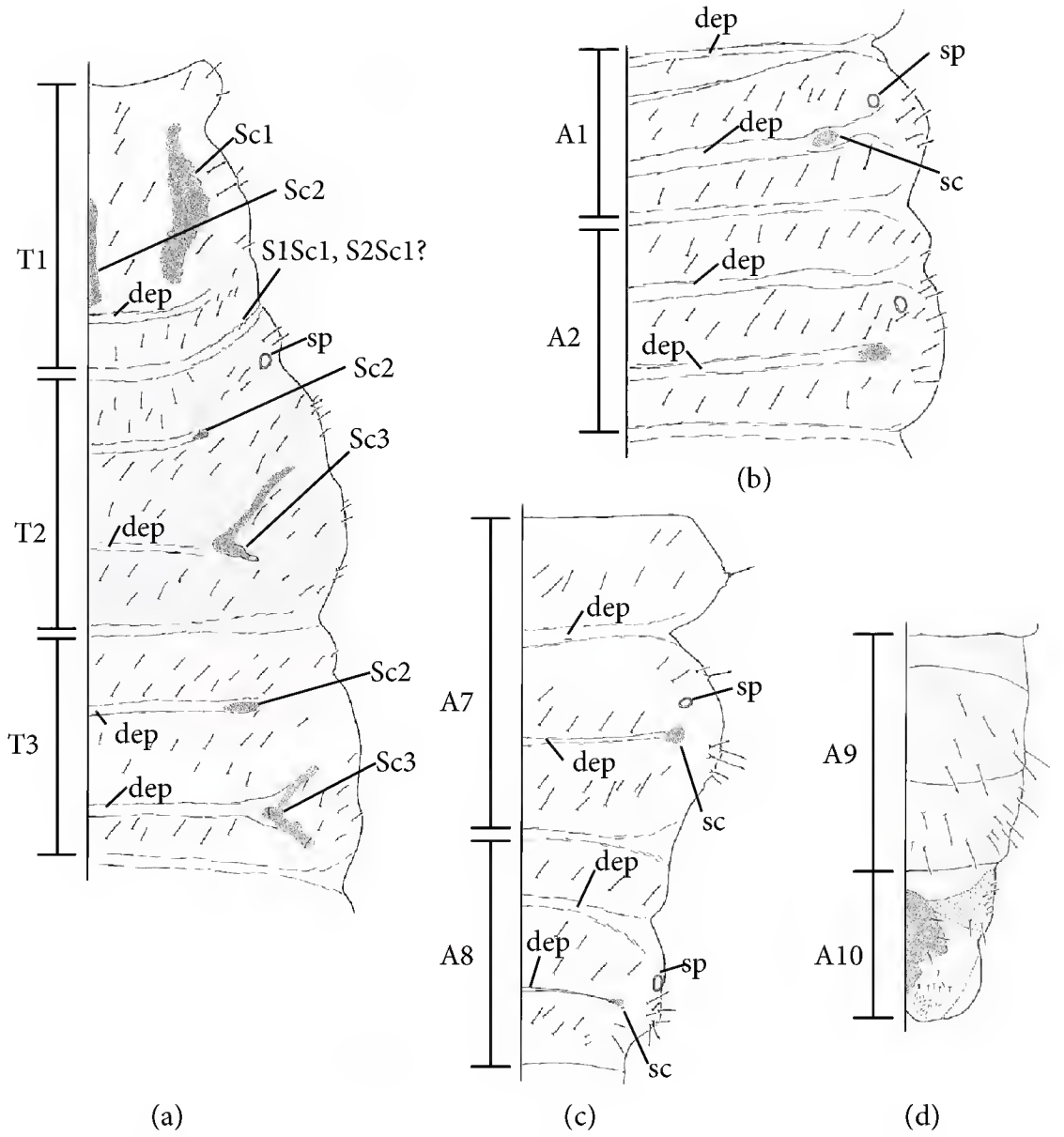

FIGURE 3: Thorax and abdomen (dorsal) of *Hypochrysa africana* Kimmins, second instar. (a) Thorax. (b) First and second abdominal segments. (c) Seventh and eighth abdominal segments. (d) Ninth and tenth abdominal segments. Abbreviations: Ax, number of abdominal segment; dep, smooth-surfaced, intrasegmental depression between subsegments; S1Sc1, S2Sc1, first and second primary setae on Sc1, Sc2, and Sc3, the first, second, and third primary sclerites of each segment; sc, abdominal sclerite; sp, spiracle; Tx, number of thoracic segment.

subsegment with pair of small setae on anterior margin (probably S1Sc1, S2Sc), but Sc1 not distinguished; spiracles simple, sessile, circular, brown, with cylindrical, tapering atrium. Second subsegment with pair of small sclerites (Sc2) on anterior margin, each with single, very small seta (S1Sc2); subsegment separated mesally from posterior subsegment by transverse depression with pair of large, bifurcating sclerites (Sc3) laterally. Sc3 with anterior arm extending anterolaterally, posterior arm extending posterolaterally, each toward edge of subsegment; center of Sc3 marked with brown, embedded in lateral stripe, entirely delineated by cream-colored area.

Metathorax (T3) consisting of three well delineated subsegments separated by transverse depressions each with pair of transversely elongated sclerites (Sc2, Sc3) laterally. Sc3 large, with bifurcating arms—anterior arm extending anterolaterally, posterior arm extending posterolaterally, each toward edge of segment; center of sclerite marked with brown, embedded in lateral stripe, entirely delineated by cream-colored area.

3.2.5. Abdomen (Figures 3(b)–3(d)). Lateral tubercles (LTs), laterodorsal tubercles (LDTs) absent; spiracles (A1–A8) circular, sessile, with simple, cylindrical, tapering atrium. Each segment (A1–A7) divided into three subsegments separated by transverse depressions; spiracle located laterally on dorsum of second subsegment; second, third subsegments separated by pronounced depression bearing pair of clear sublateral sclerites. A1: anterior subsegment small, spindle shaped, separated from second subsegment by small depression; dorsum with single pair of SMS; second, third subsegments longer, broader, similar in size and setation to those of A2, A3. A2–A8: subsegments roughly quadrangular, of similar size, extending to margin of segment, separated from adjoining subsegments by pronounced depressions; dorsum of each subsegment with five (four on anterior subsegment of A8) to 14 pairs of SMS. A4–A8: pleural region (dorsal region of second and third subsegments) with cluster of four to seven short setae. A9: cylindrical, with three subsegments (dorsal view); anterior subsegment without setae; posterior two subsegments sclerotized, with setae; mesal subsegment with ~seven pairs of dorsal setae; posterior subsegment with ~12 pairs of dorsal setae, about half of them lateral. A10: dorsum with transverse brown band, separated mesally by large arrow-shaped, darker brown mark; segment with ~five pairs of setae, numerous microsetae. All segments with few small "simple" or "denticulate" setae. Venter with subsegmentation

lightly demarcated (A1–A4) or without demarcation (A5–A9); each segment with numerous "simple" setae, usually longer than dorsal setae.

4. Discussion

4.1. Synonymy Based on Larval Characters. A comparison of the *K. africana* and *H. elegans* larvae (Semaphorant B) does not reveal any significant, generic-level differences; for comparison, see [8]. Indeed, the larvae of the two species appear to differ largely in their head markings. Because of their similarity, I consider them congeneric, and hereby, *Kimochrysa* Tjeder becomes a junior (subjective) synonym of *Hypochrysa* Hagen *(New Synonymy)*. The type species of the genus is *Chrysopa nobilis* Schneider, and the species under study here reverts to its original name, *Hypochrysa africana* Kimmins.

Given this generic synonymy, the questions now are as follows. (i) Is this synonymy, which is based on larval characters, also supported by adult morphological characters? And, (ii) how does the synonymy affect current understandings of relationships among the genera within Nothochrysinae?

4.2. Support from Adult Morphology? Of the several adult characters purported to differentiate *Kimochrysa* and *Hypochrysa*, two tend to support the synonymy; one weakly contradicts it, and three are neutral (either variable or without sufficient comparative information) as follows:

> consistent: wing venation [21]; spiracle of eighth abdominal segment (female)—opening on the eighth tergite versus on membrane (see [4]; also see Table 1).
>
> contradictory: ninth tergite and ectoproct (male and female)—fused versus separate (see [1, 4, 21]; also see Table 1).
>
> neutral: ectoproct (male)—with versus without a long, slender "appendage" (apodeme) [21]; subgenitale (female)—sclerotized versus unsclerotized [21]; microtholi (domelike cuticular glands) on male abdomen present versus absent (see [4], also see Table 1).

Below I discuss the perspectives of two sets of authors who provided evidence for separating the genera: (i) Tjeder [21] and (ii) Brooks and Barnard [2] and Brooks [4].

4.2.1. Tjeder's Perspective. Tjeder [21] described *Kimochrysa* on the basis of adult specimens of three South African species. His generic description noted similarities in the wing venation of *Kimochrysa* and *Hypochrysa*, but he felt that significant differences in their male terminalia required placing them in separate genera. Specifically, he mentioned (i) tergite 9+ectoproct—separate in *Kimochrysa* (as opposed to fused in *Hypochrysa*, both sexes), (ii) male ectoproct in *Kimochrysa*—lacking a long, slender "appendage" (probably the apodeme) that occurs in *Hypochrysa*, and (iii) subgenitale (female)—unsclerotized in *Kimochrysa* (as opposed to sclerotized in *Hypochrysa*).

Of Tjeder's above three characters, subsequent studies have shown that the first continues to provide the strongest (albeit weak) support for the separation of *Kimochrysa* from *Hypochrysa*. In males of *H. elegans* the T9+ect is fused, and in *Kimochrysa impar* (Tjeder) they are separate (males are undescribed for the other two *Kimochrysa* species) (Table 1). In females, the structures are partially fused in *H. elegans* and separate in all three species of *Kimochrysa*. It should be noted that, within at least two genera of Nothochrysinae (*Pimachrysa* Adams (males) and *Nothochrysa* (females)), this character is known to express interspecific variation. Consequently, given the small number of studied species within *Hypochrysa* and *Kimochrysa* (males: $n = 1$ for each genus; females: $n = 4$, with one showing an intermediate condition) (Table 1), this character lends only weak support for separating the two genera.

The second character (an elongate male ectoproct) is reported from *H. elegans* [1, 23] but not from any other species in Nothochrysinae [2, 4]. Thus, although this character distinguishes *H. elegans* males from the single species of *Kimochrysa* whose males are studied, its phylogenetic value at the generic-level (versus species-level) remains open.

The third character (sclerotized subgenitale) also is of questionable value at this time. Tjeder illustrated the tips of the subgenitale of the three *Kimochrysa* species. However, he was not specific about what he meant by the character and he did not include equivalent drawings of the *Hypochrysa* subgenitale for comparison. Subsequent drawings and descriptions by other authors neither provide comparative information, nor mention any differences between the subgenitale of *Hypochrysa* and *Kimochrysa* [2, 23]. Second, the character state of the genitale has not been reported for species in other genera of Nothochrysinae for meaningful comparison. In sum, the generic-level value of the character needs further evaluation.

4.2.2. Brooks and Barnard's Perspective. In subsequent studies, Brooks and Barnard [2] and Brooks [4] retained *Kimochrysa*'s distinction as a genus, apparently based on three characters. First, like Tjeder [21], they noted that the condition of the ninth tergite and ectoproct differentiated *Hypochrysa* (fused) from *Kimochrysa* (whose structures they considered partially fused). As stated above, this character currently has little informative value regarding the synonymy.

The second character was the presence or absence of microtholi on the male abdomen. This character differs between the single *Kimochrysa* species with known males (microtholi absent) and *H. elegans* (microtholi present) (Table 1). However, the condition is highly variable among the genera of Nothochrysinae both within and outside the group that contains *Hypochrysa* (Table 1). Thus, the character does not provide strong support either for or against the synonymy.

The third character concerns the placement of the spiracular opening on the female eighth abdominal segment—on

Table 1: Summary of adult characters currently used for classifying Nothochrysinae. All known species are included.

Species name	Character			
	T9 + ect*		Microtholi**	Spiracle***
	Male	Female		
Nothochrysa grouping				
Asthenochrysa				
viridula (Adams)	+ [3]	− [3, 18]	− [3]	− [3]
Dictyochrysa				
fulva Esben-Petersen†	+ [2, 19]	+ [19]	?	− [19]
latifascia Kimmins	+ [20]	?	?	+ [19]
peterseni Kimmins	+ [19]	+ [2]	?	+/− [19]
Hypochrysa				
africana Kimmins†	?	− [2, 21, 22]	?	+ [2, 21]
elegans (Burmeister)†	+ [2, 23]	+/− [2, 23]	+ [2, 23]	+ [2, 23]
impar (Tjeder)	− [2, 21]	− [21]	− [2]	+ [21]
raphidiodes (Tjeder)	?	− [21]	?	+ [21]
Nothochrysa				
californica Banks†	+ [1]	? [3]	+ [1]	?
capitata (Fabricius)†	?	+ [2]	+ [2]	?
fulviceps (Stephens)†	+ [2, 23]	− [23]	+ [2, 23]	+ [23]
indigena Needham	+ [24]	?	?	?
sinica C.–k. Yang	?	− [25]	?	+ [25]
turcica Kovanci and Canbulat	+ [26]	− [26]	?	+ [26]
Triplochrysa				
pallida Kimmins	+ [2]	− [19]	− [2]	+ [2]
kimminsi New	+ [19]	− (prob.) [19]	?	+ [19]
Pamochrysa grouping				
Leptochrysa				
prisca Adams and Penny	?	− [18]	?	− [3]
Pamochrysa				
stellata Tjeder	− [21]	− [2, 21]	− [2]	− [21]
Pimachrysa				
albocostalis Adams	− [1]	?	+ (prob.) [1]	?
fusca Adams	− [1, 2]	− [1, 2]	+ [1, 2]	− [1, 2]
grata Adams	?	− (prob.) [1]	?	?
intermedia Adams	?	− (prob.) [1]	?	?
nigra Adams	+/− [1]	− (prob.) [1]	+ [1, 2]	?

Characters: *tergite 9 and ectoproct: fused (+), unfused (−), partially fused (+/−); **microtholi on male sternites: present (+), absent (−); ***location of spiracular opening on female eighth abdominal segment: on membranous pleuron below T8 (+), on T8 (−), spanning both T8 and the membrane below (+/−); male or female unknown or character state not reported (?). †Species with larvae described. Numbers in square brackets: references.

the membrane versus on the tergite. For all know species of *Hypochrysa* and *Kimochrysa*, the spiracle opens on the membrane (Table 1); thus, it is consistent with the new synonymy.

4.3. Relationships among Genera of Nothochrysinae. The new synonymization of *Kimochrysa* with *Hypochrysa* led to a review and reevaluation of the current generic groupings within Nothochrysinae and the characters used to support the groupings. Previously, the subfamily was proposed to contain a derived "monophyletic" group of five genera (the "*Nothochrysa* group") that included *Hypochrysa* and that was supported by characters presumed to be apomorphic. The four remaining genera, including *Kimochrysa*, were considered less derived and lacked apomorphic support [4]. The present findings favor the notion that the subfamily contains two generic groupings that are similar but not identical to those listed earlier. Moreover, the strength of the "apomorphic" support for the groupings is questioned for two main reasons. First, ancestral states for the presumed apomorphies lack strong supporting evidence. Second, the characters exhibit variation within the generic groupings and sometimes even within genera.

Below is a summary of the now tentative groupings within Nothochrysinae followed by a discussion of the underlying

support and a recommendation for fuller comparative studies.

4.3.1. Groupings of Nothochrysinae Genera. Nothochrysinae is now proposed to contain two relatively distinct groupings of genera.

(i) *Nothochrysa* grouping. This category contains five genera: *Asthenochrysa* Adams, *Dictyochrysa* Esben-Petersen, *Hypochrysa* Hagen (including *Kimochrysa*), *Nothochrysa* McLachlan, and *Triplochrysa* Kimmins. Larvae have been described for three of the genera [7].

(ii) *Pamochrysa* grouping. This category contains three genera: *Leptochrysa* Adams and Penny, *Pamochrysa* Adams, and *Pimachrysa* Adams. Larvae are undescribed.

4.3.2. Supporting Characters. To provide perspective and encourage stronger comparative morphological studies, the three presumed "apomorphic characters" for these groupings are discussed below.

(i) Character number 1—ninth tergite (T9) and ectoproct of both sexes fused (versus separate). The ancestral state of this character is not determined, but an unfused condition has been considered plesiomorphic for Chrysopidae [2]. Among three neuropteran families considered to be related to Chrysopidae [5, 6, 27, 28], the T9 and ectoproct are separate in Polystoechotidae and variable (fused or unfused) in Hemerobiidae and Osmylidae [27, 29–32]. The fused character state occurs in various taxa of all three chrysopid subfamilies, including the two that are considered basal (Nothochrysinae and Apochrysinae) (for phylogenetic relationships among chrysopid subfamilies, see [5, 6]; for the distribution of morphological features, see [2, 33, 34]).

From the available literature, a fused condition occurs in males of all genera in the *Nothochrysa* grouping and in females of most genera. The condition is largely absent from both males and females of the *Pamochrysa* grouping (Table 1). The most parsimonious conclusion from the distribution of the features is that the fused T9 and ectoproct began to evolve within males of the *Pamochrysa* grouping, but that the presumed plesiomorphic state (separate T9 and ectoproct) was largely retained by both males and females. Apparently, full fusion appeared in males and fusion began in females during or soon after the differentiation of the *Nothochrysa* grouping.

The variability (especially partial fusion) in the expression of the trait may reflect differences in the degree of integumental sclerotization, rather than in the actual fusing of segments. It is well known that chrysopid adults become more sclerotized as they mature (often requiring a period of several weeks after emergence) and that their patterns of sclerotization vary considerably, individually and with age [1, 35–37]. Thus, caution is necessary in scoring and interpreting this character.

(ii) Character number 2—male sternites with microtholi being present (versus absent). Microtholi are not known from Neuroptera other than the Chrysopidae, and their absence is probably the basal state for the family. Within the Chrysopidae, microtholi are usually, but not always, absent in Apochrysinae males [2, 34], and their occurrence is highly variable among the Chrysopinae and Nothochrysinae (see [2]; also see Table 1). It is not clear why Brooks [4] considered this feature as an apomorphy for the *Nothochrysa* group, when earlier Brooks and Barnard [2] reported several genera in the group as being without microtholi and it was known that the structures occur within *Pimachrysa*. Thus, the feature's pattern of occurrence is not consistent with its identification as an apomorphy for the subfamily Nothochrysinae or for either of its proposed generic groupings.

(iii) Character number 3—female with spiracle on eighth abdominal segment opening on membrane (versus on tergite). The spiracular opening is consistently on the eighth tergite in Osmylidae [25, 27, 29, 38] and Polystoechotidae [30, 32] but its placement shows significant variation within Hemerobiidae [27, 31]. Among the Chrysopidae, the opening is on the membrane throughout the Apochrysinae and in most Chrysopinae and its placement is variable within Nothochrysinae see [4, 23, 35]; also (Table 1). The presumed, but as yet unconfirmed, plesiomorphic state for the Chrysopidae is for the eighth abdominal spiracle to open on the tergite [4].

In Nothochrysinae, a spiracular opening on the eighth female tergite typifies the *Pamochrysa* grouping; however the numbers of exemplars/genus are very small (n = 1/genus). In the *Nothochrysa* grouping, a spiracular opening on the pleural membrane appears to typify three of the five genera (*Hypochrysa*, *Nothochrysa, and Triplochrysa*), but in *Dictyochrysa* it is variable, and it is absent from the single species of *Asthenochrysa* (Table 1). An interesting intermediate situation occurs in *Dictyochrysa peterseni* Kimmins; in this species the spiracular opening appears to span the membrane and the tergite [19, Figure 66]. Thus, although Brooks may be correct in his proposal [4] that the spiracular opening on the membrane is a "stem apomorphic character" for the "*Nothochrysa* group," it appears more likely that the character evolved within the *Nothochrysa* grouping after its differentiation.

It is noteworthy that all species of the Chrysopinae subgenus *Chrysopodes* (*Neosuarius*) Adams and Penny have their spiracular opening on Tergite 8 [35]—a presumed reversal to the pleisiomorphic state. In this group, where large series of specimens are available for comparative study, there appears to be developmental and interspecific variation in the extent and intensity of the tergite's lateral sclerotization, so that, in some specimens, especially those that are teneral, the spiracle appears to open on an unsclerotized portion of the pleuron membrane. If a similar situation occurs in the Nothochrysinae, it could present a confounding factor similar to that in Character number 1 above.

4.3.3. Future Studies. From the above, it is evident that the few adult characters currently used to explain the phylogeny of Nothochrysinae (the presumed basal chrysopid group) offer interesting but extremely limited information on the evolutionary history of the group and of the subfamily. Efforts to improve this situation have been hampered, in large part,

by the lack of specimens (both adult and larval) for many of the rare, crucial taxa. It is hoped that the discussion here helps stimulate efforts to collect and study this ancient group so that future studies will be based on a broad ranging set of characters (from comparative adult and larval morphology, molecular studies, and comparative biology) and the full range of taxa.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research Article

Evidence for the Absence of Worker Behavioral Subcastes in the Sociobiologically Primitive Australian Ant *Nothomyrmecia macrops* Clark (Hymenoptera: Formicidae: Myrmeciinae)

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Activity in three colonies of the nocturnally foraging Australian ant *Nothomyrmecia macrops* is investigated. Workers apprehended while foraging were marked, released, and later recaptured within nests following excavation. *Every* forager in each nest was encountered and marked. It was expected that unmarked, nonforaging, domestic-specialist workers would be discovered in the nests. This was unexpectedly not the case as all workers, apart from one or two in each colony, had been marked, and therefore had foraged at least once during the three-night experiment. The few unmarked individuals are considered to have been temporarily residential nest-entrance guards. Behavioral subcastes comprising "domestic" *versus* "foraging" workers were thus not indicated, evidencing absence of worker caste polyethism in *Nothomyrmecia*. The experiment predated emergence in the nests of adult workers from cocoon-enclosed pupae at a season when large feeding larvae of the current annual brood were still being provisioned by foragers. Because *Nothomyrmecia* is univoltine and emergence of current-brood adults had not yet occurred, all workers present were from preceding annual broods and defined as "postjuvenile." A previous laboratory study separately evidenced absence of polyethism in *Nothomyrmecia*. Relevance of the apparent absence of food sharing in *N. macrops* is discussed.

Dedicated to the memory of Roger Bartell (1940–1985) esteemed friend and colleague in much Nothomyrmecia field work

1. Introduction

Following evolution of the gyne/queen and worker castes in ants, task specialization (division of labor or polyethism) among workers would likely early have involved distinction between (1) *intranidal* domestic functions and (2) *extranidal* food-collecting activities. This could encourage situations where workers operated *either* as domestics or as foragers, (1) intermittently, (2) in age-based succession, or (3) in either role essentially for life (thus comprising two behavioral subcastes exemplifying *caste polyethism*) (see [1–5]).

The experiment reviewed here investigated the relativities of intranidal versus extranidal activity among workers in three colonies of the Australian endemic Dinosaur Ant *Nothomyrmecia macrops* Clark (subfamily Myrmeciinae), seeking to determine whether or not dedicated domestic versus specialist foraging subcastes were differentiated among *Nothomyrmecia* workers. The negative alternative was clearly evidenced.

Phylogenetically *N. macrops* is a member of the myrmeciomorph group of subfamilies in the formicoid clade [6, 7]. It appears to have retained many behavioural and sociobiological traits considered ancestral for ants as a whole [8, 9], and it lacks many of the sociobiologically advanced features known in other ants [8, 9].

The absence of caste polyethism has been previously reported in only one other ant species, the "sociobiologically

primitive" *Stigmatomma pallipes* (Haldeman) (Amblyoponinae) [10] (discussed there as *Amblyopone pallipes,* nomenclature here follows Yoshimura and Fisher [11]). Alternatively, the *Nothomyrmecia*-related, evidentially more sociobiologically derived, myrmeciines *Myrmecia brevinoda* Forel [12], and *M. gulosa* (Fabricius) have bimodally size-ranged polymorphic workers. In *M. gulosa* colonies the smallest workers associate with the queen, eggs and small larvae [13], and apparently never leave the nests [14 and *pers. obs*]. They appear to constitute a nest-bound non-foraging domestic worker sub-caste.

Most ants, including *Nothomyrmecia, Myrmecia,* and *Stigmatomma,* maintain perennial univoltine colonies [9]. In general, a single annual brood is reared from eggs laid by queens during a few weeks in spring. Development to adulthood takes around 12 months. The growing larvae are eventually overwintered and begin to pupate the following spring, about a year after oviposition, at around the time eggs of the next generation are being laid. Adult workers eclose from pupation (prior to escaping pupal cocoons in the above genera) during the late spring and summer, concluding at around the autumn equinox. For this reason the presence or absence and relative proportions of eggs, hatchling, and other small larvae, large larvae, and pupae vary seasonally, and there is a long autumn/winter period during which larvae of a single generation alone are present as brood in nests (and during which there is very little extranidal activity by workers, apparently because the larvae do not feed in winter, and prey collection is suspended [9]). Colonies in spring contain eggs and recently hatched small larvae of the latest generation, along with large previously overwintered old larvae and pupae of the preceding generation, with egg numbers declining as hatching proceeds and large-larval numbers reducing as pupation progresses. As the season advances workers begin to eclose and escape from their pupal cocoons, and all emerge by late summer or early autumn, leaving only part-grown larvae of the latest generation which are subsequently overwintered. These factors dictated the seasonal timing of the experiment described here.

Univoltinism results in recruitment of a single cohort of newly emerged workers each year during summer and early autumn, covering the time from beginning to end of eclosion of adults from pupation. Because of this, workers in mature colonies fall into discrete, usually morphologically unidentifiable, age-cohorts, each on average approximately one year younger than its predecessor. The number of worker generations present in any particular nest is determined by individual longevity and the age of the resident colony. Individuals of the earliest broods, including the first (initially laid down by queens at colony foundation), would often probably not survive to be represented in older colonies.

"Juvenile" *Nothomyrmecia* workers are defined here as adults under several months of posteclosion age, which are too young to have experienced the winter following their emergence versus "postjuvenile" adult workers, comprising older, previously overwintered, individuals surviving in colonies from former annual brood cohorts. Significance of the first overwintering experience involves the theoretical possibility that any (if any) polyethism among juvenile workers would possibly be lost during their first experience of winter colony dormancy, when foraging for prey by workers and behavioral acts related to eggs, actively feeding larvae or pupae are suspended.

The *Nothomyrmecia* colonies studied here were considered to be at least 3 or 4 years old (based on their worker numbers, and that their experimentally marked active nest entrances had been noted approximately one and two years previously). Whether their oldest workers were survivors from the initial founding brood is not known. *All* workers present, however, would have been postjuvenile and more than 10 or 11 months old, given that the current nearly mature *ca* 1-year-old annual brood comprised large larvae and cocoon-enclosed pupae and had not yet produced (prospectively juvenile) adults. Two, three, or more annual age cohorts are estimated to have been represented.

The experiment was conducted in mid-November, 48–54 days after the vernal equinox, in Mallee *Eucalyptus* woodland at 85 m elevation, just south of Poochera, South Australia, about 600 m ESE of the Eyre Highway/Streaky Bay Road junction, at estimated coordinates 32°43′26.43″S and 134°50′21.78″E.

2. Experimental Assumptions

The following characteristics of *Nothomyrmecia* were assumed when designing the experiment or relate to discussion of its results. All have been repeatedly observed or experimentally demonstrated. (See also references [8, 9, 15–17].)

(1) Nests function independently of each other and there is no morphological polymorphism among workers. (2) Each nest has a single, obscure entrance hole about 4-5 mm in diameter. (3) At all times, night and day, single guard workers may be observed inside nest entrances. They emerge briefly following insertion of a disturbing filament or a live ant of another species. (4) Foraging activity is strictly nocturnal. With the exception of assumption 11 below, *Nothomyrmecia* workers have never been observed naturally abroad in daylight. Departing foragers cross the ground promptly at dusk to ascend host trees on which they remain until returning directly across the ground at dawn to home nests. (5) Foragers depart nests *en masse* at dusk, emerging from the entrance, often partly queuing briefly in close single files, over a period of 10 to 20 minutes beginning at about the time they cease to be discernable by naked eye. (6) Each forager "head-scans" on leaving the nest entrance, apparently confirming or recording visual navigational landmarks. The head, which is normally held parallel to the substrate, is pivoted slowly from side to side over an angle of about 80–100 degrees for up to 20 seconds while the ant stands otherwise motionless (often still partly within the nest entrance) with the antennal scapes held symmetrically, enclosing an angle of about 120 degrees. (7) Departing foragers disturbed experimentally near nest entrances after initial head scanning move away, rarely retreating back into the nest. (8) Prolonged uninterrupted liquid feeding for personal nutrition (terminated and presumably satiated by full crop distension) by foragers at

interceptive diluted honey baits smeared experimentally on host tree trunks (Figure 2) does not suppress their behavioral drive to continue hunting for prey. Unless they eventually capture prey, these ants do not return to the nests until dawn. (9) Successful huntresses return to home nests during the night carrying the undissected prey in their jaws (Figure 3). (10) Unsuccessful huntress foragers return to nests at dawn. Their numbers usually greatly exceed those of previously returned successful foragers. The appearance of first-light apparently cancels their prey-getting drive, initiating nest return. Arrival at home nests is concentrated over 25–40 minutes from first light to approximately when the ants are faintly visible without artificial illumination. (11) Returnees failing to reach home nests before being overtaken by daylight secrete themselves in leaf litter near tree bases or ground-level bark crevices. And presumably return eventually to their nests. (12) *Nothomyrmecia* queens leave nests to forage during colony foundation, probably up to the time when their first daughter workers appear. They are not known to exit the nests of worker-right colonies. (13) All adult ants are liquid feeders [9, pages 592-593]. *Nothomyrmecia* workers seek and imbibe appropriate nourishment while foraging outside the nests. Captured prey is returned to nests without dissection, largely as food for the carnivorous larvae (with some in-nest liquid feeding on hemolymph labiated from the remains of larval-dissected prey by workers and by the queen). (14) *Nothomyrmecia* workers do not lay chemical scent trails, and navigation is entirely visual.

FIGURE 1: *Nothomyrmecia macrops* Clark: prospective foragers impounded at dusk under experimental cover dish prior to colour marking. The nest entrance is at the top of the picture (Poochera, SA, nest "yellow," R. W. Taylor).

3. Experimental Procedure

(1) Foraging workers departing an active *Nothomyrmecia* field nest were intercepted by covering the nest entrance with a large (ca. 190 mm dia., 20 mm deep) inverted clear glass evaporation dish (Figure 1). This was placed during the day and revisited after dark following accumulation under its cover of prospective foragers which had emerged from the nest entrance at dusk. (2) All detained ants were collected, carried together to a nearby field caravan, and promptly marked with single mesosomal or gastral paint spots of a single color (using diluted Tipp-Ex typists correction fluid) applied with a fine artists brush under a low-power stereomicroscope. Specimens were collected by aspiration and handled using soft spring-steel forceps. They were not anaesthetized or chilled. The marked ants were then promptly released as a group within 10 cm of the uncovered nest entrance. (3) After all had dispersed the cover dish was replaced over the nest entrance and checked regularly during the night and at dawn for returned foragers standing on its upturned base or at its periphery. Any previously marked returnees were replaced under the dish. The cover was removed in the morning after the last returnees had accumulated. Returnees without color spots were intercepted and color marked. (4) These procedures continued nightly until all ants seen on two successive dusk-to-dawn periods were observed to have been previously color marked. (5) The nest was then fully excavated and all resident ants collected. (6) The numbers of marked and unmarked ants were recorded. (7) The experiment was simultaneously replicated on three adjacent colonies situated in a triangle with sides approximately 3 M long; workers from each colony were separately distinguished by white, yellow, or green paint marks.

4. Experimental Results

(1) All ants replaced near nest entrances after marking promptly performed head scans and moved off to forage. None were observed to return immediately to their nests. (2) Morning-marking was required for only one forager returning at dawn on day 2 (colony "green"). It was presumably overlooked the previous evening or had secreted itself during the previous day after failed colony return. (3) The numbers of successful foragers returning with prey during the night were low: 2 for colonies "white" and "green" on night 1, 1 for colony "yellow" on night 1, and 2 on night 2 (about 5.1% of the total worker count). The great majority of returnees accumulated outside the covering dishes during the dawn return period without prey. (4) Single nest-entrance guards were observed in all colonies both by day and at night while the foragers were abroad. None were collected for color marking for fear of disrupting their behavior. (5) The marking

FIGURE 2: *Nothomyrmecia macrops* Clark: foraging workers feeding nocturnally at experimental honey bait on host *Eucalyptus* tree trunk. These ants accumulated as individuals, without collaborative interaction. Note the progressively distended gasters from right to left. (Poochera, SA, R. W. Taylor).

FIGURE 3: *Nothomyrmecia macrops* Clark: foraging worker with prey (male psyllid bug, Hemiptera: Psyllidae) (nocturnal, Poochera, SA, Ajay Narendra).

of workers was completed on night 3 for all three colonies. All those accumulated under the cover dishes at dusk or on their surfaces or surrounds at dawn on nights 4 and 5 were observed to have been previously marked, terminating the experiment. (6) The colonies were excavated and tallied on day 6. (7) The frequency of marked versus unmarked workers collected from each colony is given in Table 1. (8) All recovered colonies contained only one queen. All contained the appropriate seasonal brood of old, near fully grown larvae and congenerational cocoon-enclosed pupae of the current annual generation, with small, young larvae of the following year's cohort, destined to complete development to adulthood about 10–12 months later. There were no recognizable newly emerged callow workers or evidence (such as freshly cast pupal cocoons in nest middens) that eclosion had recently occurred. (9) No marked workers were recovered from nests other than those at which they were marked. (10) Queens were not observed to leave the nests.

If hard-wired caste polyethism was present in the subject colonies, dedicated domestic workers would not have departed the nests as foragers and would have been retrieved unmarked at excavation. This was not the case. The experimental results instead convincingly demonstrate that postjuvenile workers in mature, actively foraging field colonies of *Nothomyrmecia macrops* during the peak annual prey-getting season regularly engage in foraging and that any intranidal activity by individuals generally does not persist continuously for more than a few days before being interrupted by foraging. In the three nests (1) 41–50% of workers foraged on the first night; (2) 28–37% of workers first foraged after at least 1 night spent in the nest; (3) 13–25% of workers first foraged after at least 2 nights spent in the nest. The consistency of results suggests that workers generally do not spend more than two nights in nests between foraging expeditions. During the three-night observation period 96–98% left the nests to forage. The interception of previously marked workers on nights 2 and 3 indicates departures by individuals on successive nights. Four unmarked workers (2.9% of the 137 in all 3 colonies) evidently did not leave the nests during 5 nights prior to excavation.

The unmarked workers were probably nest-entrance guards. The observed presence of guards during the experiment supports this conclusion. The assumption that guards are temporarily excused from foraging is tenable. Supporting evidence is provided by Jaisson et al. [16]. It is unlikely that these ants were foragers previously overlooked for marking or newly emerged juvenile workers not yet behaviorally programmed to forage. They might have been members of a queen retinue. The numbers indicate, however, that all but one or two workers of any such group present on day 1 must have foraged during the experiment (even assuming that the stayers were not guards or juveniles).

Experimental procedure prevented successful foragers from departing on a second same-night foray (they were inserted under the cover dish when apprehended in its vicinity with prey and so restricted to the nest for the remainder of the night). It is therefore not known if repeat foraging is practiced by *Nothomyrmecia*. By comparison, about 10% of individually numbered foragers of the phylogenetically related night-active bulldog ant *Myrmecia pyriformis* Smith have been reported to depart their nest repeatedly (usually twice, but up to 4 times in one individual) in a 1-night observation period [17].

In *Nothomyrmecia* (and *Myrmecia pyriformis* [17]) visual navigation across the ground between nest and host tree in dusk and dawn light is essentially crepuscular, and vision is probably less important than geotaxis for navigation on host trees during full darkness [15]. Nonetheless, nest return across the ground by prey-laden foragers in full nocturnal darkness clearly demonstrates capacity for visual navigation under such conditions. The *Nothomyrmecia* field program did not routinely record lunar phase, though for at least some nights of peak activity the Poochera site is clearly recalled to have been brightly moonlit. Future researchers should note this detail (see [18, 19]).

The experiment would have been more informative if different sets of color marks had been used each night and foragers marked or remarked so as to record individual 5-night foraging histories. Also, the entrance guards should perhaps have been extracted and marked with separate identifying colors.

Repetition of the experiment later in the season after emergence of juvenile workers would indicate whether there is a delay preceding foraging activity by juveniles after eclosion, because many more unmarked workers would then

Table 1: The numbers of previously unmarked foraging workers departing 3 *Nothomyrmecia macrops* nests on 3 consecutive experimental nights, with the total numbers present in each nest as determined by a later excavation. Percentages are those of the full tallies for day 6.

Colony	White	Yellow	Green
Night 1	19 (41.3%)	26 (50.0%)	18 (46.1%)
Night 2	17 (37.9%)	18 (34.6%)	11 (28.9%)
Night 3	8 (17.3%)	7 (13.4%)	10 (25.2%)
Totals	44 (95.6%)	51 (98.0%)	38 (97.4%)
Excavated (day 6)	**46** (44 + 2) (100%)	**52** (51 + 1) (100%)	**39** (38 + 1) (100%)

be expected to remain in the nests during the experimental period. If colonies were marked as described here prior to the eclosion season and excavated weeks later following the emergence of juveniles, marked postjuvenile and unmarked juvenile workers would be distinguishable. Similar use of a cover dish to intercept returning foragers could provide frequency estimates of successful versus unsuccessful prey-collection departures and facilitate identification of prey organisms.

5. Observation Nest Studies

Social organization in two laboratory-based queen-right observation colonies of *N. macrops* with individually numbered workers was reported by Jaisson et al. [16].

The colonies were at approximately the same stage of annual brood cycle as those used in the field experiment discussed above, with a cohort of large larvae and pupae but no congenerational recently emerged workers, along with eggs and small larvae of a younger cohort. All workers must have been more than 10-11 months old.

Results demonstrated that (1) there is a strong tendency by the ants not to engage in social interaction (interactive acts were less than 1% of the 4,002 recorded) and (2) very few workers performed queen-directed acts. Some temporarily located near the queen more often than others, but were seldom in direct contact. "Their high co-presence indices...indicate...that they constitute a functional "royal retinue";" (3) worker polyethism was otherwise not apparent. Some individuals showed a propensity to guard the nest entrance. This activity "evidenced a high degree of specialization among its executors." Three workers performed as guards as well as foraging and caring for larvae in Colony 1, while one was involved at high frequency, and seven at very low frequency in Colony 2. None of the latter engaged in foraging, which in any case was relatively infrequent in colony 2; (4) exchange of food directly by trophallaxis or as trophic eggs was not observed between workers, workers and queen, or adults and larvae, apart from worker placement of intact, undissected prey near the larvae, which tore intersegmental membranes and dismembered prey carcasses by their own feeding activity; (5) the queen was observed to feed on hemolymph without assistance from workers, by licking larval-predissected prey; (6) there was no evidence of a dichotomy between dedicated domestic and foraging worker subcastes nor was there evidence in this study that workers specialized long term in performing particular in-nest activities, so it is reasonable to assume that this was also the case within the nests investigated in the field study reported here.

6. Discussion and Conclusions

All available evidence indicates that division of labor is likely absent in postjuvenile *Nothomyrmecia macrops* workers, apart from slight tendencies for one or two individuals at any one time to act as nest-entrance guards or several as members of a royal retinue. These acts are temporary and do not divert participants for long from other tasks including foraging and brood tending. Individual service in the queen retinue is short-lived judging from the laboratory study and field results in which so few workers remained in nests over the 5 subject nights.

Because their brood cycles antedated emergence of workers from cocoons, neither the field nor laboratory colonies discussed contained juvenile adult workers (as defined above). Future observation-nest investigation of juvenile-worker-right colonies could facilitate recognition (or not) of behavioral differences among juveniles or between juveniles and postjuveniles and the possibility (or not) of identifying such behavior as hard-wired age-based temporal polyethism. Until this is investigated it cannot be affirmed that inherently programmed polyethism is absent among *Nothomyrmecia* workers, despite the evidence of this account and Jaisson et al. [16]. That is the reason why juvenile workers have been distinguished and defined here. Future researchers will need carefully to consider the findings of Traniello and associates [1, 2].

There is no evidence for intergenerational polyethism between workers of different annual brood cohorts, or among similarly aged workers of any single postjuvenile generational cohort nor, in the absence of anatomical polymorphism, is there any other evidence of caste polyethism among *Nothomyrmecia* workers.

The *Stigmatomma pallipes* colonies studied by Traniello [10] contained both juvenile and postjuvenile workers. The former, recognizable as relatively recently emerged callows, engaged in both brood-tending and foraging similarly to older (postjuvenile) noncallow individuals, except that they were not involved in egg care. Some individuals showed a degree of attachment to particular tasks, but this did not exclude them from other actions, and none of the acts

under observation were significantly correlated. Traniello and Rosengaus [20] rightly point out that in these "primitive" ants "foraging is part of brood-care activity because larvae are directly provisioned with prey, and the same worker performs both tasks," and so it is with *Nothomyrmecia*. *Nothomyrmecia* workers operate almost entirely "in the manner of solitary insects" (to use Wilson's expression [4]). Alleged behaviorally programmed (as opposed to chance) interactions or cooperation between noncallow workers is extremely rare [15, 16]. Interactions between *Nothomyrmecia* individuals are predominantly those between workers and brood [16]. It would be difficult to envisage less-collegial adult ants!

Exchange of food between workers, workers and queen, or adults and larvae by trophallaxis or as trophic eggs (as in *Myrmecia* [21, 22]) was not observed in *Nothomyrmecia* by Jaisson et al. [16] or by the author and others during many hours of focused or casual observation of laboratory colonies. Neither has larval hemolymph feeding been observed, as in *Stigmatomma oregonense* Wheeler [23] (cited there as *Amblyopone oregonensis*), a species close to *S. pallipes*. The ability secondarily to distribute nourishment therefore appears to be lacking in *N. macrops*, which presumably might never have evolutionarily acquired this capacity (secondary loss of such valuable behavior seems unlikely). This is evidently yet another "primitive" sociobiological characteristic of *Nothomyrmecia*. Workers, like queens, lick hemolymph from prey in nests following its dismemberment by feeding larvae. When foraging, they feed at experimental honey baits for up to 45 minutes while their gasters expand visibly due to crop distension (Figure 2). They normally lick carbohydrate-rich psyllid lerps, honeydew, and other sugary materials on leaves.

The specific need for workers to depart nests seeking liquid or soluble food for personal nourishment is exacerbated by the apparent absence of food-sharing behavior. Workers are known also to drink nocturnally at rainwater droplets accumulated in concave dry leaves lying on the ground (pers. obs.).

It could be argued that the prospect for further evolutionary sociobiological progression in *N. macrops* is compromised because food is not secondarily distributed within its colonies. For example, support of a domestic worker subcaste confined more-or-less permanently within nests might be functionally untenable, since its members might not receive adequate nourishment without personal foraging (though nest-bound queens clearly do survive and function effectively, apparently on prey hemolymph, perhaps on that alone in the apparent absence of food sharing). It is of interest that foraging workers, whatever their motivation for nest departure (whether personal hunger or stimulation by hungry larvae), engage in prey-hunting following personal satiation at experimental liquid-food baits. Thus, in terms of both personal feeding, and obtaining food for larvae, all departures are maximally utilized.

Conflict of Interests

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

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Research Article

Immature Stages and Life Cycle of the Wasp Moth, *Cosmosoma auge* (Lepidoptera: Erebidae: Arctiinae) under Laboratory Conditions

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Cosmosoma auge (Linnaeus 1767) (Lepidoptera: Erebidae) is a Neotropical arctiid moth common in Cuban mountainous areas; however, its life cycle remains unknown. In this work, *C. auge* life cycle is described for the first time; also, immature stages are described using a Cuban population. Larvae were obtained from gravid wild females caught in Viñales National Park and were fed with fresh leaves of its host plant, the climbing hempweed *Mikania micrantha* Kunth (Asterales: Asteraceae), which is a new host plant record. Eggs are hemispherical and hatching occurred five days after laying. Larval period had six instars and lasted between 20 and 22 days. First and last larval stages are easily distinguishable from others. First stage has body covered by chalazae and last stage has body covered by verrucae as other stages but has a tuft on each side of A1 and A7. Eggs and larvae features agree with Arctiinae pattern. Pupal stage lasted eight days, and, in general, females emerge before males as a result of pupal stage duration differences between sexes.

1. Introduction

Cosmosoma is a large Neotropical moth genus with approximately 155 species [1], and some of them are broadly distributed. Wasp moth *Cosmosoma auge* L. (Lepidoptera: Erebidae) occurs in Central America, South America, and the Caribbean islands. In Cuba, it occurs throughout the main island with big populations in the main mountainous areas. As in other moth taxa, knowledge about immature stages of this genus is lacking and there are no descriptions of their life cycle. Dyar [2] described *C. myrodora* immature stages from a Florida population and Castillo [3] described *C. myrodora* larval and pupal stages duration which were fed with three *Mikania* species, but no instar duration or larval morphology were taken into account. Wild larval ecological traits are also unknown for *Cosmosoma* species. In this paper we describe life cycle and larval stages for *Cosmosoma auge* from a Cuban population. We also report a new host plant for this species and some immature ecological traits. Other host plants reported for *C. auge* in HOSTS databases are *Cecropia peltata* (Cecropiaceae), *Ipomoea* sp. (Convolvulaceae), *Lagenaria siceraria* (Cucurbitaceae), *Mikania pachyphylla*, *M. parviflora*, and *M. scandens* (Asterales: Asteraceae) [4].

2. Materials and Methods

2.1. Collecting and Rearing. Larvae were obtained from seven wild *Cosmosoma auge* females collected at Viñales National Park in September 2011. As Peterson [5] suggested, females were kept confined together in a plastic jar until they laid eggs. Newly hatched larvae were placed in plastic Petri dishes (100 × 10 mm) and they were provided with fresh host plant leaves daily. Larvae that hatched the same day were placed together in groups of 30 individuals. When growth desynchronization occurred, larvae were sorted in other Petri dishes in order to keep the same age groups to detect individual variations in stages length. Larvae were reared in captivity at Cojimar, Eastern Havana, under natural photoperiod, humidity, and temperature between September 29th and October 21st 2011.

Daily temperature fluctuated between 24 and 28°C with average of 26°C, and the relative humidity varied from 54 to 93% with average of 79%. Both abiotic variables were provided by Casablanca Meteorological Station, Havana.

Host plant identification and ecological traits descriptions were made with botanical samples and animals from Sierra del Rosario Biosphere Reserve. Some individuals ($n = 3$) were fed with *Cecropia peltata* to prove its suitability as host plant.

2.2. Life Cycle and Description of Immature Stages. Egg laying patterns were described from wild clutches found on the host plant leaves. Duration of the egg stage was measured and proportion of infertile eggs was also recorded. Egg shape and dimension were described using eggs obtained from wild females caught in Sierra del Rosario Biosphere Reserve. Two base diameters and height were measured using an ocular micrometrer (0.05 mm precision) attached to a stereomicroscope Olympus. Egg volume ($N = 36$) was calculated following García-Barros [6]. Surface relief and micropilar details were observed with a SEM (250x magnification).

Petri dishes were checked daily for head capsule exuviae to establish larval instar duration. The head capsule width was measured (using the same ocular micrometer) directly on the larvae while resting and was defined as the distance between outermost ocelli. Two body lengths per stage were measured. One length was taken after molt (minimum length) and another in the premolt phase (maximum length), when larvae have emptied their gut and became light yellow. First and second instar larvae length were measured using the ocular micrometer mentioned above, and third to sixth instar larvae length were measured using a Vernier caliper with 0.05 mm precision.

Larvae that pupated the same day were placed together to measure the duration of the pupal stage. Pupae maximal length and width were measured using the same Vernier caliper. At emergence, adults' sex was recorded in order to detect differences in pupal stage duration among sex.

Stehr [7] and Scoble [8] were followed for morphological terminology. Photographs of larvae, pupae, and adults were taken using a macro mode of a 10.1 megapixels digital camera. Eggs, at least three individuals of each larval stage, two pupae, and cocoons were preserved in 75% alcohol and housed in Felipe Poey Natural History Museum, University of Havana. Fourth to sixth larval instars were killed in very hot water to prevent gut content decomposition.

2.3. Statistical Analysis. Mean and standard deviation of larval stages duration were calculated. Stage duration and pupal period length between sexes were compared using a randomization test from Monte Carlo algorithm, using the PopTools complement of Microsoft Excel; 10000 iterations were made.

3. Results

3.1. Host Plant and Ecological Considerations. Wild caterpillars were found feeding on climbing hempweed *Mikania micrantha* Kunth (Asterales: Asteraceae) and laboratory cohorts were successfully raised with it. Larvae fed with *Cecropia peltata* spent most of the time walking all over the Petri dish; they did not eat the leaves and died of starvation.

Table 1: Larval stages duration of *Cosmosoma auge* raised at Cojímar, Havana, under laboratory conditions (September-October 2011).

Instar	Duration (days)		Mean ± SD
1st ($N = 40$)	3 ($N = 15$)	4 ($N = 25$)	3.6 ± 0.49
2nd ($N = 40$)	2 ($N = 21$)	3 ($N = 19$)	2.5 ± 0.50
3rd ($N = 40$)	2 ($N = 13$)	3 ($N = 27$)	2.7 ± 0.47
4th ($N = 39$)	2 ($N = 8$)	3 ($N = 31$)	2.8 ± 0.44
5th ($N = 35$)	3 ($N = 22$)	4 ($N = 13$)	3.4 ± 0.59
6th ($N = 24$)	6 ($N = 7$)	7 ($N = 17$)	6.7 ± 0.46

Cosmosoma auge is a multivoltine species with multiple generations along the year; adults were caught almost every month. In the wild, eggs were laid singly ($n = 9$), in pairs ($n = 5$), and in trios ($n = 3$) on the underside of mature leaves of its host plant. Only two clusters with five eggs and two clusters with eight and ten eggs, respectively, were found. Eggs were always found in the basal portion of host plant leaves, near to veins.

First and second instar larvae fed on the leaf epidermis while other instar larvae fed on all the laminae but not on the 1st and 2nd order veins. Second instar larvae fed on shed skin as all the other larval instars did. In the wild, 4th and 6th instar larvae were found feeding on leaves underside at night. Always one larva per leaf was found. In the wild, larvae were apparently hidden during the day but in laboratory conditions larvae of all instar fed at day also. When larvae were disturbed they fell down holding themselves with a silk thread. In the wild, we found empty cocoons on the upperface and the underface of host plant leaves.

3.2. Life Cycle. A total number of 134 eggs were obtained and 47 of these (35%) did not hatch; but they were not infertile because in all cases a well-formed embryo could be seen through eggshell. Eggs ($n = 87$) hatched days after oviposition at any time of day five. Larval stage lasted 20 to 22 (21.5 ± 0.66) days and pupae ended their development 8 to 10 (8.6 ± 0.56) days later. Instar's duration was between two and four days for immature larvae and six or seven days for last instar larvae (Table 1). Only one larva had a four-day 4th instar period, and two larvae had two-day and five-day 5th instar, respectively. There were differences in larval stage duration: 1st and 5th instar lasted longer than 2nd, 3rd, and 4th instar ($P < 0.001$), and the 6th instar lasted significantly longer than all the previous instars ($P \leq 0.003$). Most of the 40 larvae that completed their life cycle reached the 6th instar; only three of them reached the 7th instar and were not included in this analysis.

At the end of the larval phase, the larvae have a quiescent prepupal phase, which lasted about two days, and afterward they entered in the pupal stage. Pupal mortality was 15% of the 43 pupae considered. Adult emersion ($n = 35$) occurred at any time of the day, although most of it occurred in

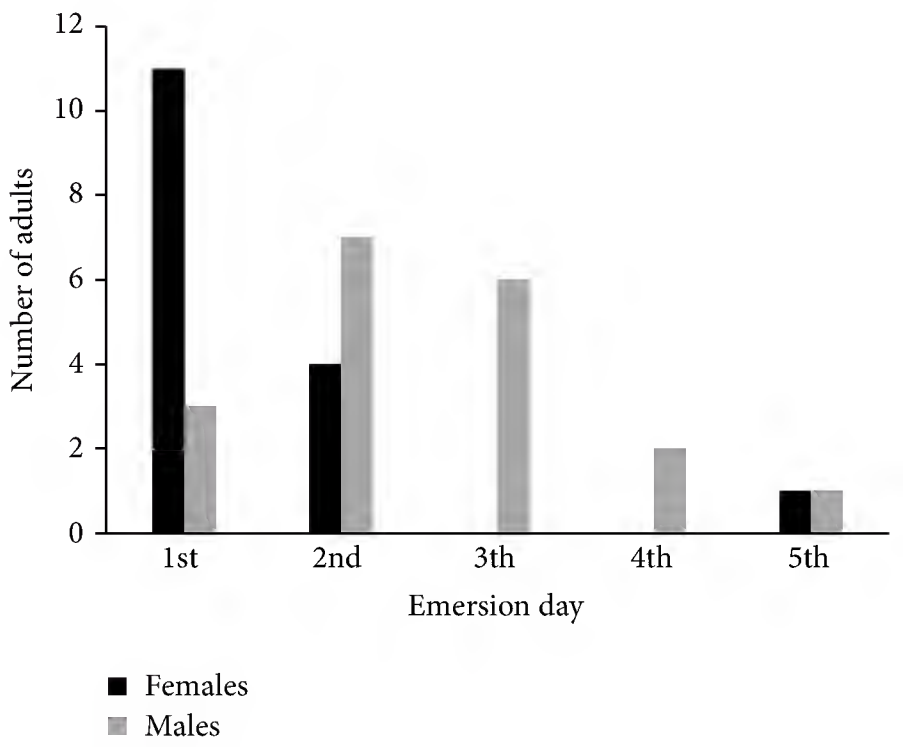


FIGURE 1: Number of *Cosmosoma auge* imagoes (both sexes) that emerged in each emersion day. Specimens were raised under laboratory conditions at Cojímar, Havana (September-October 2011).

the afternoon hours ($n = 29$). Pupal stage lasted eight to ten days. Females ($n = 16$) had a one day shorter pupal period than males ($n = 19$) ($P \leq 0.0032$). There were differences in emersion dynamics between sexes as a consequence of their differences in pupal period. The 68% of total females emerged in the first emersion day while males didn't reach the 50% of adults emerged in the second emersion day (Figure 1). These results suggest that *C. auge* presents protogyny. Adult lifespan was 15 days maximum ($n = 1$).

3.3. Immature Stages. Eggs are hemispherical and upright, with 0.87 ± 0.05 mm diameter and 0.62 ± 0.05 mm height. Eggs volume was 0.25 ± 0.028 mm^3. The base was not flat but concave. Egg color is ivory due to its content; all thought the transparent and shiny eggshell gives a pearly appearance (Figure 2(a)). There is no color change during development, though on eclosion day, the larva mandibles can be seen beneath the eggshell as two pale brown spots that are close together and mobile. Chorion was hexagonally and pentagonally reticulated (Figure 2(b)) except for the micropilar area, which was rosette-like, composed by five petaloid cells (Figure 2(c)). In the eclosion day, the only difference was the presence of two tiny, mobile, and pale brown spots (larva mandibulae) on the egg surface.

First instar larvae: minimum length 2.65 ± 0.25 mm ($n = 62$) and maximum length 3.75 ± 0.20 mm ($n = 34$), and head capsule width 0.40 ± 0.05 mm ($n = 53$). Body covered by chalazae and dorsal chalazae longer than lateral ones. Six stemmata in two rows, four dorsal stemmata forming a semicircle, and two ventral stemmata separated from dorsal ones by a distance equal to stemmata diameter. Prolegs with heteroideus crochets in a mesoseries, distal lobe with 10 big hooks in the middle and many little hooks in both sides. Body color was ivory, almost white, but A8, which was light yellow dorsally. Thorax and abdomen turn green when larvae feed because of their translucent body wall (Figure 3(a)). Head is yellow, but stemmata are black and distal edge of mandibles is reddish brown. Heads have white and tiny setae. Larvae had some two-tone dorsal setae (black base and white tip) interspersed with white regular setae. Rarely, these bicolored setae were predominant on larva's body, which looks grayish. All larval stages have bare venter.

Second instar larvae: minimum length 4.10 ± 0.40 mm ($n = 43$) and maximum length 5.80 ± 0.65 mm ($n = 48$). Head capsule width was 0.50 ± 0.05 mm ($n = 45$). The main change in this instar is that chalazae are replaced by verrucae except the chalazae in the anterior edge of T1 which were oriented forward. Thorax segments T2 and T3 and abdominal segments were covered by verrucae. Verrucae with many white setae and two or three black and long setae. Legs and prolegs had white tiny setae. Setae in T2, T3, and A1 are longer than the other body's setae. There were no significant color changes from first instar (Figure 3(b)) in this or next stages.

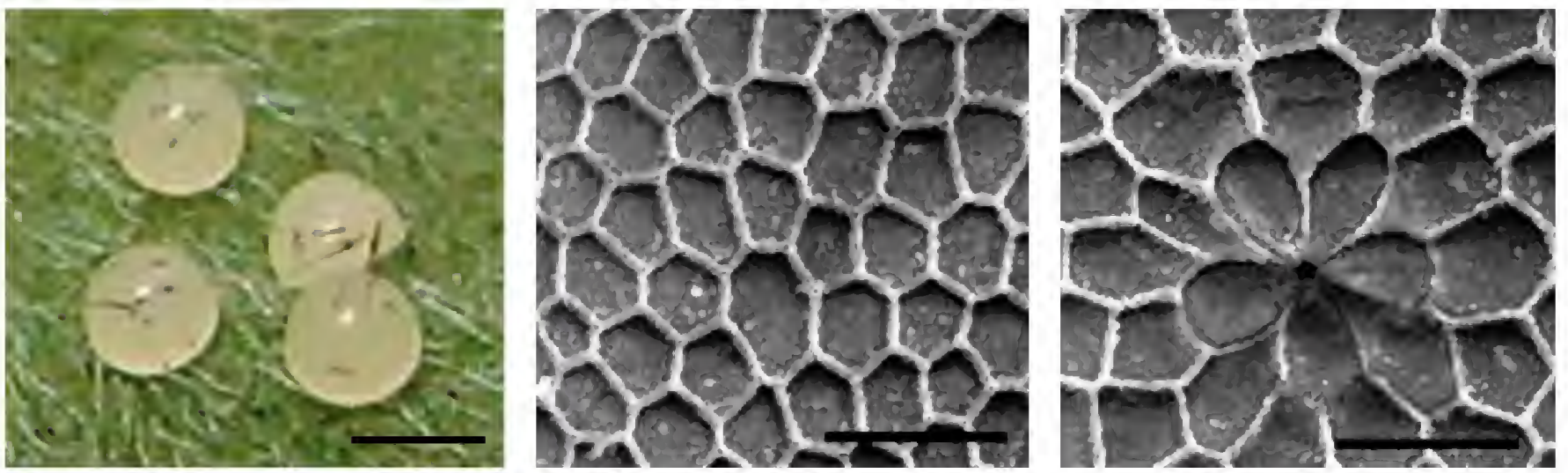
Third instar larvae: minimum length 7.80 ± 0.60 mm ($n = 8$) and maximum length 8.35 ± 0.45 mm ($n = 18$). Head capsule width was 0.75 ± 0.05 mm ($n = 13$). There are five black chalazae in the anterior edge of T1 oriented forward. Body covered by verrucae, included T1. Verrucae had several black setae in T2, T3, and A1–A8.

Fourth instar larvae: minimum length 9.30 ± 1.45 mm ($n = 23$) and maximum length 11.65 ± 0.50 mm ($n = 26$). Head capsule width was 1.05 ± 0.05 mm ($n = 15$). Body covered by verrucae with white and black setae. Dorsal verrucae with some long setae and lateral verrucae with setae shorter than dorsal ones. Legs and prolegs covered by short, white, and thin setae. Oval spiracle in T1 and A1–A8. Head with some tiny setae.

Fifth instar larvae: maximum length 15.60 ± 0.90 mm ($n = 28$). Head capsule width was 1.45 ± 0.05 mm ($n = 21$). Body covered by verrucae with white and black setae. Legs and prolegs have white and tiny setae. Spiracles in T1 and A1 had medium size, A8 spiracle is big and conspicuous, the rest are tiny.

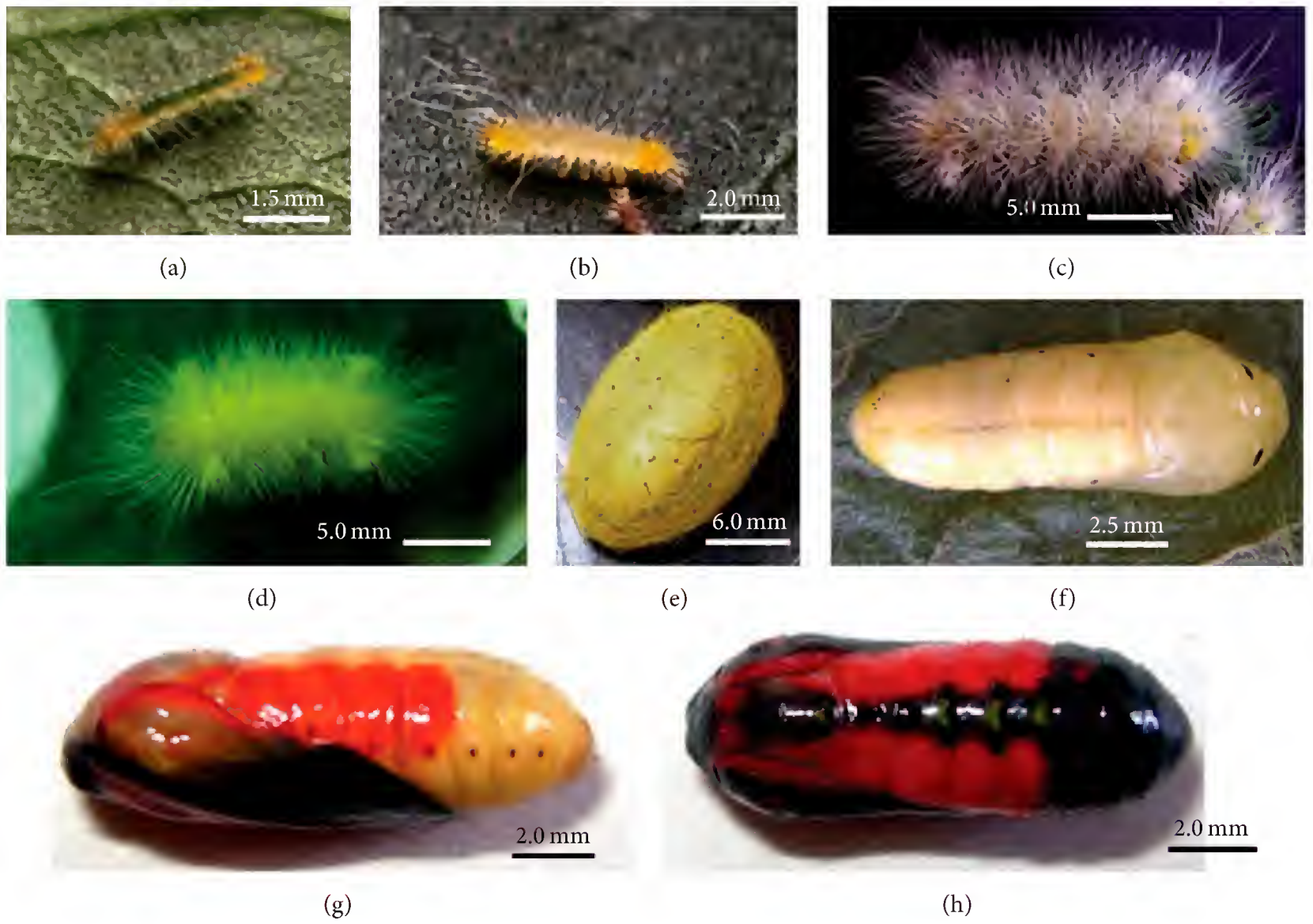
Sixth instar larvae: minimum length 15.40 ± 1.30 mm ($n = 35$) and maximum length 21.60 ± 2.00 mm ($n = 22$). Head capsule width was 1.95 ± 0.05 mm ($n = 16$). At the end of the larval stage they reached eight times their initial length at hatch and about 30% of this growth occurs during the last instar because of an increased growth rate (Figure 4). Mature larva's body was covered by verrucae with white setae, T2, T3, and A1 verrucae with some black setae. A1 and A7 had a pair of white tuft located laterally perpendicular to longitudinal body axis (Figure 3(c)). Most of the tuft setae are white but it had some black plumed setae. There are gray setae in A8 and A9. Also, in A8 there are two bright yellow spots dorsally, one in each side of heart.

In the prepupal phase, the mature larvae stopped feeding and searched for a place to pupate. Then, they remained quiet, emptied their gut, and turned bright yellow, especially setae (Figure 3(d)). Only white setae changed color, black setae remained the same. Afterward they shrank and detached setae from their bodies to construct cocoons mixing them with silk. The denuded larvae are light yellow and they keep that way until early pupal stage.



(a) (b) (c)

FIGURE 2: *Cosmosoma auge* eggs. General view (a), chorion surface detail (b), micropilar area (c). Scale bar represents 1 mm (a) and 500 μm ((b) and (c)).



(a) (b) (c)

(d) (e) (f)

(g) (h)

FIGURE 3: Some immature stages and cocoon of *Cosmosoma auge*. First instar larva (a), second instar larva (b), last instar larva (c), prepupa (d), cocoon with pupa inside (e), recently molted pupa, dorsal view (f), pupa 12 hours before emersion, lateral view (g), and pupa just before emersion, dorsal view (h).

Pupae: obtect and enclosed in a thin, walled, and bright yellow ellipsoidal cocoon (Figure 3(e)). Pupae length is 12.45 ± 0.65 mm (n = 43) and width is 4.60 ± 0.25 mm (n = 42). Abdominal segments with punctures moderately dense on dorsal part. Few setae dispersed on the lateral part close to the spiracles and also on dorsal part. Ventrally, the wing tips reach the A4 segment. The cremaster is weak and consisting of three rows of translucent hook-like setae only visible with a magnification of 40x. Pupae color is shiny pale yellow, head and thorax are translucent yellow but abdomen was whitish yellow. Newly formed pupae have two little dark spots on anterior mesonotum, which correspond with thoracic spiracles (Figure 3(f)). Eyes (only a line) and spiracles are brown, which quickly became black. After four days, eyes became brown oval areas and in the 5th day, they became black. In the 7th day, joints are light brown. In the 8th day, head and thorax as well as two stripes on the abdominal dorsum from A1 to A4 turn orange red (Figure 3(g)); wings and the four rear segments as well as a middorsal stripe on the thorax and abdomen between the red stripes are dark

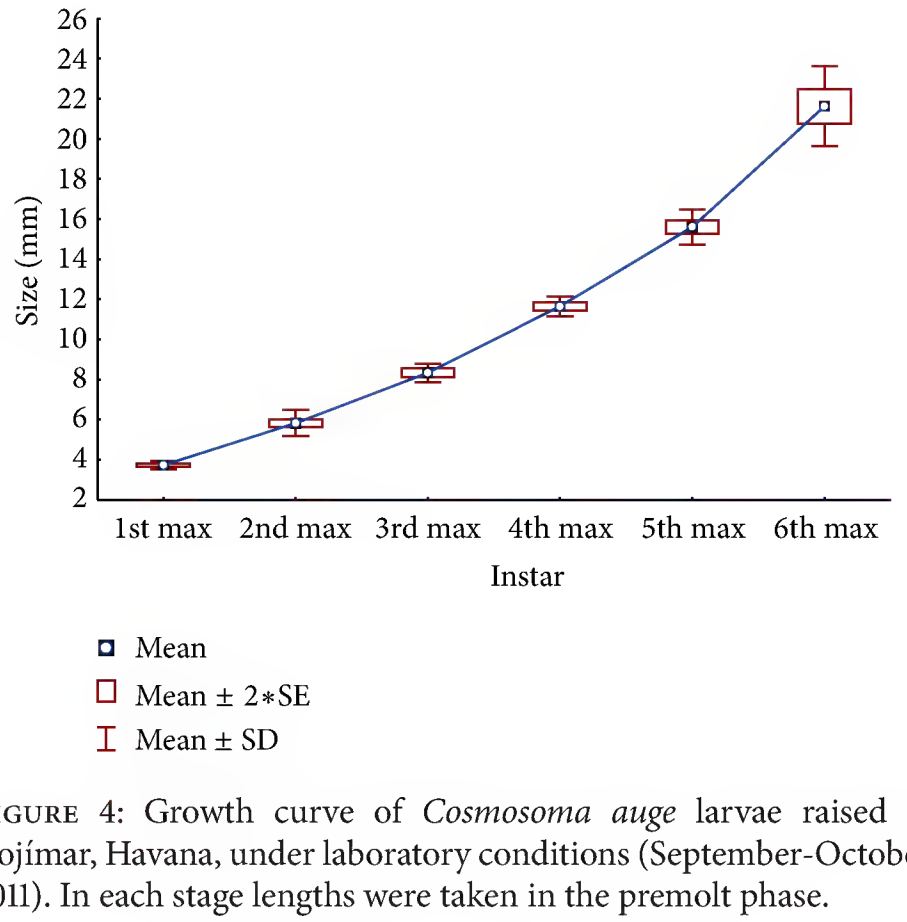


Figure 4: Growth curve of *Cosmosoma auge* larvae raised at Cojímar, Havana, under laboratory conditions (September-October 2011). In each stage lengths were taken in the premolt phase.

gray. Just before emersion these gray zones turn black with dark blue iridescence (Figure 3(h)). Pupae show adult's color and looked shiny at that point. After that, it turned opaque because pupae skin separates from imago within it.

4. Discussion

Mikania micrantha is the first host plant reported for *C. auge* in Cuba and belongs to the same genus of *M. scandens*, host plant reported for North American *C. myrodora* [9]. It is known that Lepidoptera phytophagous larvae had a strong specificity for a host plant family or even genus [10]. As Thompson and Pellmyr [11] state, females are capable of discrimination among plant species, host plant genotypes, and host plants in different microhabitats. The record of *Cecropia peltata* as *C. auge*'s host plant could be a misidentification of larvae feeding on it. Also, it could be a result of taxonomic problems in *C. auge*, in case of being a complex group cryptic species.

The finding of wild small clutches and solitary eggs, as in Florida's *C. myrodora* [2], suggests that *C. auge* has a solitary larval strategy in concordance with Uvarov [12] definition. Wild larvae feeding pattern also reinforces this hypothesis. Larval growth under different density conditions was studied for this species (León-Finalé and Barro unpublished data) and results agree with the hypothesis of larva solitary strategy in *C. auge*. According to Stamp [13], species with solitary larva reduced the probabilities of been found by parasitoids and predators, and also reduced food and pupation sites competition. Matsumoto [14] states that grouped larvae are at risk of suffering starvation due to host plant defoliation if plant is small or isolated. *Mikania micrantha* is a vine with limited leaves number and *C. auge* larvae are voracious in later stages. A large number of individuals could potentially defoliate a single plant quickly. Occasionally, *M. micrantha* grows grouped and there is potentially more food available for larvae. Females could detect this unusual availability of larval food [11] and laid larger clusters of eggs (eight or ten), as we detect in Sierra del Rosario population. All these arguments make a study to define *C. auge* larval strategy necessary.

Eggs shape, surface relief, and color were similar to Florida's *C. myrodora* eggs [2, 5]. Eggs are fairly uniform in structure throughout the subfamily [7]. Dyar [2] found that *C. myrodora* had an egg period length of eight days but we found an egg period of five days. This difference could a result of differences in abiotic parameters, as temperature. Time and size traits are affected by temperature, photoperiod, humidity, diet, density, and host quality [15]. Also this could be a product of inherent differences between *C. auge* and *C. myrodora*.

As a whole, morphology of immature stages of *C. auge* is similar to general arctiid pattern [7, 8] and is very constant along non-first instar larvae in this species, without major changes in color or morphology. As in *Phoenicoprocta capistrata* [16], the first instar is only covered by chalazae bearing one long filiform seta and the intermediate instars are covered by verrucae. The last instar (6th) was morphologically recognizable from the previous ones for the presence of one pair of tufts in A1 and A7. The drastic color change in the prepupal period could be a result of accumulation of colored substances in setae, in case of *C. auge* having transparent and hollow setae. The larvae features are basically the same of *C. myrodora* [2] but we observed six instars in almost all the cases instead of seven, also the head widths of all instars were larger in this work, but the seventh stage mature larvae in Dyar [2] have a wider head capsule than sixth stage mature larvae of *C. auge*.

In *C. auge*, as in other Lepidoptera species [17–20], last instar is where maximal growth and reserve accumulation occurred, because it was the only instar that lasted more than four days and the larvae grew around 30% of their total length. In this case, the last instar could be a critical period determinant of adult size, fecundity, and lifespan. The adult size could be determinant in sexual selection [21], mate frequency [22], and number of eggs laid by females [23].

Life cycle of *C. auge* was similar to but more dynamic than other Arctiinae species, probably as a result of an intense feeding behaviour [24, 25], because larvae were feeding during day and night [7]. Besides *C. auge*, there are differences in pupal period between sexes in other Arctiinae species as *Empyreuma pugione, Dyauxes ancilla,* and *Phoenicoprocta capistrata* [16, 26, 27]. In those, as in *C. auge,* males emerge after females. Differences in emersion rate could be a result of sex specific needs in gonadal maturation or a strategy to avoid inbreeding [28]. Also, *C. auge* time lapse between the start of females and males' sexual receptivity could be even longer if males consume pyrrolizidine alkaloids after the emersion as *C. myrodora* males do [9].

5. Conclusions

Cosmosoma auge life cycle was quite similar to that described for other Arctiinae species but was much more dynamic probably as a result of intense feeding behavior. Larvae are very constant in color and shape but pupae, on the other

hand, are brightly colored and variable, on the contrary of most other arctiid moths.

Conflict of Interests

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

Acknowledgments

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Research Article

Report on a Large Collection of *Merope tuber* Newman, 1838 (Mecoptera: Meropeidae), from Arkansas, with Notes on Collection Technique, Sex Ratio, and Male Clasper Size

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A large collection of earwigflies, *Merope tuber*, is reported from Arkansas, and flight period and sex ratio are discussed. In contrast to previous studies, earwigflies were caught more frequently in pan traps than in Malaise traps and male clasper size was found not to be bimodal.

1. Introduction

Merope tuber Newman, 1838, known as earwigflies or forcepflies, are uncommonly collected and have fascinated entomologists since their discovery in 1837 (Figure 1). This fascination was initially due to their presumed rarity—only 16 specimens were collected between their discovery and 1904 [1]. Since then, they have continued to receive attention due to their previously assumed basal phylogenetic position within Mecoptera, relatively unknown life history, undescribed larvae, and odd appearance relative to other Mecoptera (e.g., flattened body, opisthognathous head, and broad wings folded over the abdomen) [2, 3].

Only two other extant meropeids exist: *Austromerope poultoni* Killington, 1933 [4], from Western Australia and *Austromerope brasiliensis* Machado et al., 2013 [3], from Brazil. One extinct species, *Boreomerope antiqua* Novokschonov, 1995 [5], is known from Middle Jurassic lacustrine claystone near Kubekovo Village in Siberia. Four extinct species of *Thaumatomerope* (i.e., *T. madygenica* Rasnitsyn, 1974, *T. minuta* Rasnitsyn, 1974, *T. oligoneura* Rasnitsyn, 1974, and *T. sogdiana* Rasnitsyn, 1974) were originally assigned to Meropeidae but were later reassigned to Thaumatomeropidae [6, 7].

Collections of *M. tuber* continue to be infrequent. Prior to 1954, it was reported only from areas in or east of the Appalachian Mountains. Since then, the known range has been extended north to southern Ontario [8–10], west to Minnesota [11, 12], Iowa [13], Missouri [14–16], Arkansas [13, 16, 17], and Kansas [13], and south to Alabama [18], Georgia [17], and Florida [19, 20]. Rather than true emigration, this range expansion is best explained by the increased use of various passive trapping techniques [14]. *Merope tuber* have been collected using Malaise traps, picric acid traps, European chafer traps, carbon dioxide traps, molasses traps, and glue traps [2, 12, 21], with the most effective being Malaise traps [22].

Little is known about the life history of *M. tuber*. Adults are nocturnal and attracted to light at night and spend daylight hours under logs and stones [1, 21]. They seem to be associated with moist deciduous woodlands near water [21, 23], although they are occasionally caught in dry grasslands far from any stream or creek [10]. Feeding preferences are unknown, although they may be attracted to carrion [2] similar to another mecopteran, *Notiothauma reedi* McLachlan, 1877, which has been reported from vertebrate carrion [24]. Adults stridulate by rubbing the jugum of the forewing against the metanotum [25]. The larvae of all meropeids, including *M. tuber*, remain undescribed [26] and their discovery "is certainly the most exciting thing left to be done in the study of North American Mecoptera" [14].

Figure 1: *Merope tuber*, male.

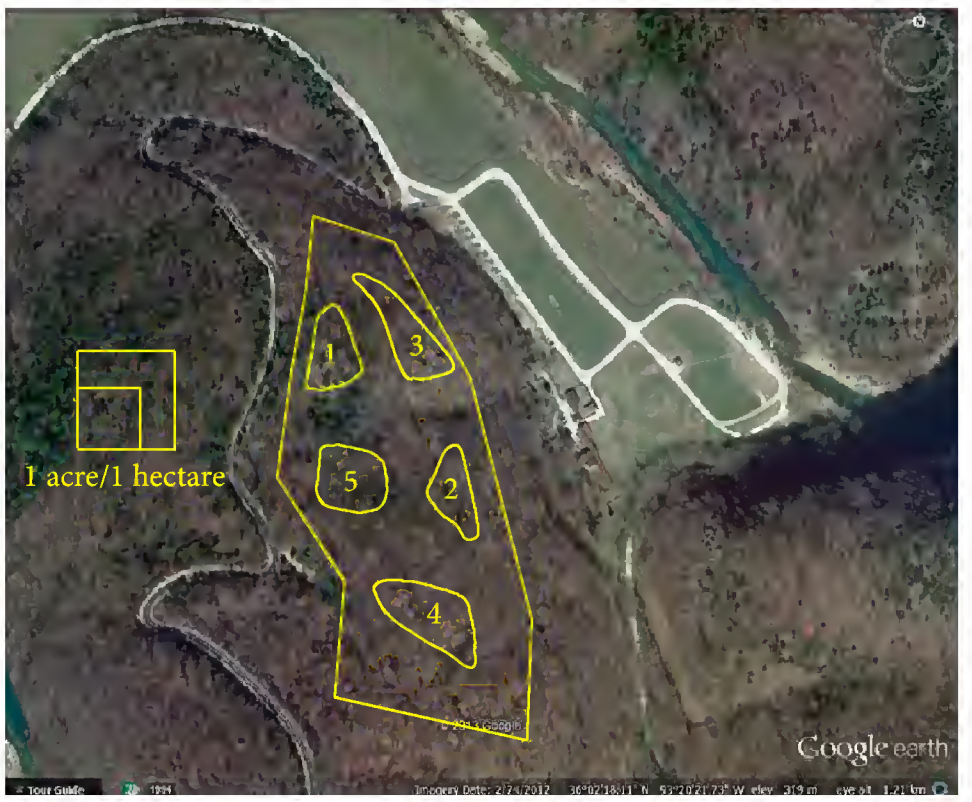

Figure 2: Overhead view of the field site at Steel Creek, with approximate limits of the site and blocks and acre/hectare scales in yellow. Base image taken from Google Earth [29].

The flight period of *M. tuber* lasts throughout the summer with some variation depending on latitude. They have been reported to occur in June through October in Connecticut [27], June through September in Maryland [28], July through September in Ohio [26], May through September in Alabama [18], and April through December in Florida [19, 20].

Few studies have reported *M. tuber* in significant numbers, but, in those that do, the sex ratio appears to be female biased. Scarbrough [30] collected 8 males and 18 females (1 male : 2.25 females) in two Malaise traps over a period of three years. Maier [27] collected 26 males and 43 females (1 male : 1.65 females) in a single Malaise trap over three years. Barrows and Flint [28], in six Malaise traps over the course of seven months, caught no males and 35 females. Johnson [26], in a single Malaise trap over two years, caught 61 males and 102 females (1 male : 1.67 females), the largest number of earwigflies yet reported from a single site. It is not known whether the sex ratio is truly skewed or if sampling bias is the cause.

Unlike life history, much is known about the morphology of *M. tuber*, with both internal and external anatomy of both sexes being well documented [31–34]. Males have elongated genital styli (= claspers) that are thought to be used in mating

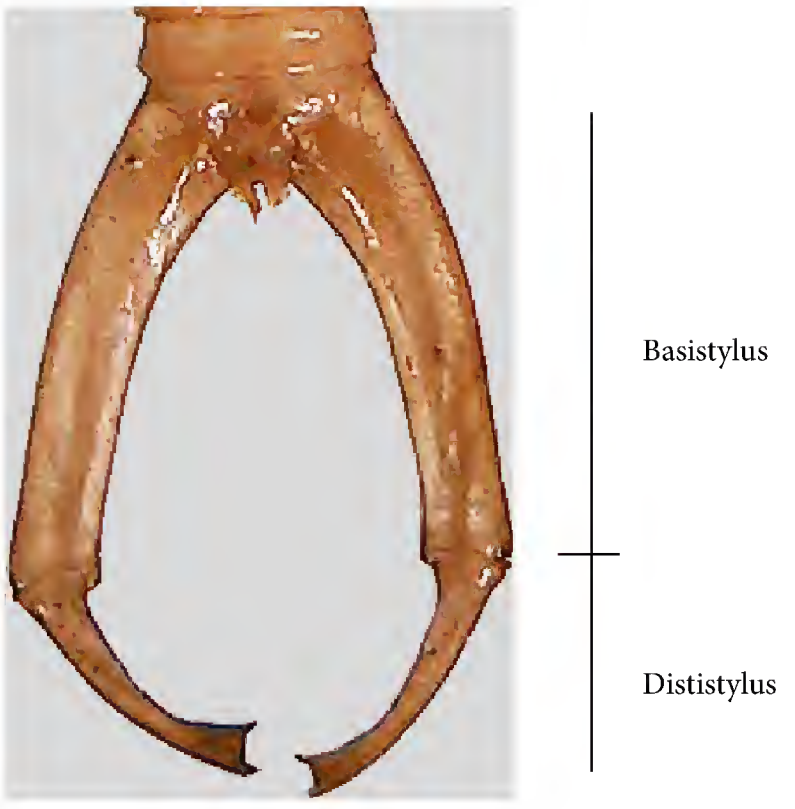

Figure 3: Clasper of male *Merope tuber* with basistylus and dististylus labeled.

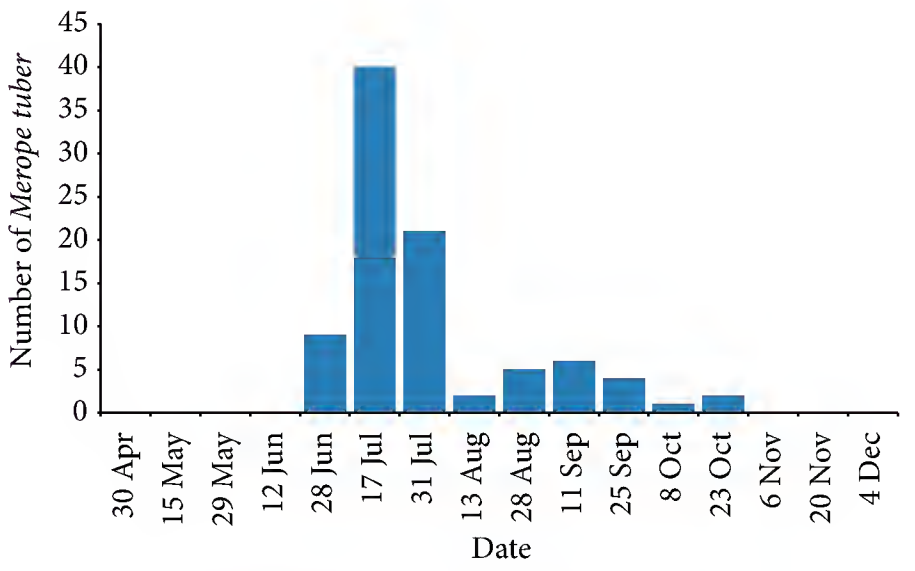

Figure 4: Number of *Merope tuber* collected across all traps per date.

as in other Mecoptera, either holding the female during copulation, fighting rival males, or both [26]. A bimodal distribution in clasper size has been demonstrated for at least one population with differential mating strategies being suggested as a possible cause [26].

2. Materials and Methods

As part of a more extensive arthropod sampling project, five blocks were established at a 4 ha plot located at Steel Creek along the Buffalo National River in Arkansas (Figure 2). In each block, five pan traps (one of each color: blue, red, green, yellow, and white) were randomly arranged under a terrestrial Malaise trap (MegaView Science Co., Ltd., Taichung, Taiwan), which was placed in perceived flight paths. In addition, three Lindgren funnel traps (ChemTica Internacional, S.A., Heredia, Costa Rica) (one of each color: green, purple, and black) were suspended nonrandomly from large trees 4–10 meters from the ground in the lower canopy.

Table 1: Total number of *Merope tuber* collected per trap type per block, with subtotals of trap type and block.

	Block	Number of females caught	Number of males caught	Total caught
Trap type				
Malaise trap	1	0	0	0
Pan trap (purple)	1	1	1	2
Pan trap (yellow)	1	1	0	1
Pan trap (blue)	1	0	0	0
Pan trap (white)	1	1	0	1
Pan trap (red)	1	0	0	0
Malaise trap	2	0	1	1
Pan trap (purple)	2	2	0	2
Pan trap (yellow)	2	1	0	1
Pan trap (blue)	2	2	1	3
Pan trap (white)	2	2	1	3
Pan trap (red)	2	4	1	5
Malaise trap	3	0	0	0
Pan trap (purple)	3	2	0	2
Pan trap (yellow)	3	0	0	0
Pan trap (blue)	3	0	1	1
Pan trap (white)	3	1	0	1
Pan trap (red)	3	1	1	2
Malaise trap	4	0	0	0
Pan trap (purple)	4	5	3	8
Pan trap (yellow)	4	8	2	10
Pan trap (blue)	4	7	3	10
Pan trap (white)	4	2	2	4
Pan trap (red)	4	2	1	3
Malaise trap	5	1	0	1
Pan trap (purple)	5	2	3	5
Pan trap (yellow)	5	5	1	6
Pan trap (blue)	5	2	1	3
Pan trap (white)	5	4	0	4
Pan trap (red)	5	2	1	3
Trap subtotal				
Malaise trap	—	1	1	2
Pan trap (purple)	—	12	7	19
Pan trap (yellow)	—	15	3	18
Pan trap (blue)	—	11	6	17
Pan trap (white)	—	10	3	13
Pan trap (red)	—	9	4	13
Block subtotal				
—	1	3	1	4
—	2	11	4	15
—	3	4	2	6
—	4	24	11	35
—	5	16	6	22
Total	—	**58**	**24**	**82**

Table 2: Minimum, maximum, and mean measurements of various body parts and results of Shapiro-Wilk goodness-of-fit tests on the same. $P < 0.05$ is considered significant. Significant values are indicated by an asterisk (*).

Measurement	Sex	Minimum (mm)	Maximum (mm)	Mean (mm)	SD (mm)	W	Prob. < W
Head width	Female	0.8	1.32	1.1	0.12	0.97	0.247
Pronotum width	Female	1.06	1.69	1.41	0.16	0.97	0.196
Forewing length	Female	8.86	13.28	11.66	0.9	0.98	0.337
Abdomen length	Female	4.1	8.96	6.44	1.3	0.97	0.153
Head width	Male	0.77	1.39	1.11	0.15	0.96	0.534
Pronotum width	Male	0.95	1.63	1.31	0.17	0.97	0.756
Forewing length	Male	9.52	13.39	11.82	1.04	0.971	0.695
Abdomen length	Male	4.07	7.61	5.8	0.78	0.95	0.206
Basistylus length	Male	2.21	5.09	4.05	0.77	0.95	0.265
Dististylus length	Male	1.47	2.91	2.34	0.43	0.91	0.036*
Clasper total length	Male	3.68	7.97	6.38	1.17	0.94	0.138

Four blocks contained a SLAM (Sea, Land, and Air Malaise, MegaView Science Co., Ltd., Taichung, Taiwan) trap (with top and bottom collectors counted as separate traps). Three blocks contained pitfall trap sets placed every five meters along a transect centered on a Malaise trap. Two of these blocks contained eight pitfall trap sets and one block contained a single set.

Pitfall traps were modified from a design proposed by Nordlander [35], which Lemieux and Lindgren [36] demonstrated that it catches carabids in similar numbers but is more efficient at excluding small vertebrate bycatch. Rather than cutting circular entrances in the sides of pitfall traps, we cut three slots, 2 cm tall × 9.3 cm wide and 2 cm under the rim in the sides of plastic soup containers leaving three 1.5 cm posts, equidistant apart, resulting in a 28 cm collecting surface. Diameter at the base of slots is approximately 10.5 cm and the cups are 10.5 cm deep below these slots, resulting in a collecting volume of 2,988 cm^3. This allowed the matching lid to be secured to the cup instead of using a separate cover. A single cup was placed on either side of a 30.5 cm × 15.5 cm aluminum fence to make a pitfall trap set and the catch from both cups was combined and treated as a single sample.

Propylene glycol (Peak RV & Marine Antifreeze) (Old World Industries, LLC, Northbrook, IL) was used as a preservative in all trap types. Traps were placed on March 13, 2013, taken down on December 4, 2013, and collected approximately every two weeks. Trap catch was sieved in the field and stored in Whirl-Pak bags (Nasco, Fort Atkinson, WI) in 90% ethanol until sorting. After sorting, specimens were stored individually in 2 mL microtubes (VWR International, LLC, Randor, PA) in 70% ethanol. Voucher specimens have been submitted to the University of Arkansas Arthropod Museum.

Head width, pronotum width, wing length, and abdomen length were measured for both sexes. The lengths of the basistylus and dististylus (Figure 3) were measured on the right side of males and combined to measure total clasper length.

Measurements were made in the following manner: photographs of a millimeter ruler and dorsal and ventral aspect of each specimen were taken through the eye piece of a Leica MZ 16 stereomicroscope with the camera on an HTC Droid Incredible 4 G LTE; zoom was not adjusted between photographs to ensure they were to the same scale. All photographs were exported onto a desktop computer, opened in ImageJ [37], and measurements were taken by tracing the structures. Measurements were recorded in Microsoft Excel (Redmond, WA).

Shapiro-Wilk goodness-of-fit tests ($\alpha = 0.05$) were performed in JMP (SAS Institute, Cary, NC) to test normality of previously described measurements. An F-test for significance was performed by creating a generalized linear model (GLM) with a Gaussian distribution ($\alpha = 0.05$). Count data were not normally distributed and required transformation. Because the data contained many zeroes, one was added to each count and before a natural log transformation. Because five pan traps were placed with a single Malaise trap, trap types could not be compared due to extremely skewed sample sizes. Instead, Malaise traps were considered a "color" in analyses and tested against each pan trap color. This simultaneously allowed for comparisons among variables of equal sample sizes for both trap type and pan color.

3. Results and Conclusions

All totaled eighty-two earwigflies—24 males and 58 females (1 male : 2.42 females)—were collected (Table 1). This female-biased collection is in line with previous studies [26–28, 30]. Earwigflies were first collected in late June, with the largest collection occurring in July, followed by low, but consistent, numbers caught until late October (Figure 4). The beginning and end of the flight period were consistent with other areas at similar latitudes [19, 26–28].

Only a single body measurement, the dististylus, differed significantly from a normal distribution, but not in a bimodal manner (Table 2). These results are in contrast to previous studies, which found a bimodal distribution in the size of male basistyli, dististyli, and total clasper length [26]. As the use of the claspers is unknown, the significance of this is also unknown.

Earwigflies were not caught in SLAM traps, Lindgren funnel traps, or pitfall trap sets; therefore, these traps were excluded from analyses. Significantly fewer *M. tuber* were caught in Malaise traps compared to pan traps ($t = -2.455$,

d.f. = 1, P = 0.0145), although pan trap colors were not significantly different from each other. This is the first report of earwigflies being collected in pan traps; however, previous studies which reported large collections of *M. tuber* traditionally used Malaise traps alone. It should be noted that, because pan traps were directly under Malaise traps, it is unknown whether those pan trap-collected individuals would have been captured in the Malaise trap collecting head, had pan traps not been present.

Significantly more earwigflies were caught in blocks 4 (t = 4.307, d.f. = 1, P = 0.00002) and 5 (t = 2.479, d.f. = 1, P = 0.0136) than in blocks 1, 2, and 3. This suggests that trap placement and microhabitat, even within a relatively small area of a few hectares, are important factors when collecting earwigflies. If earwigflies are specifically targeted, we suggest placing multiple traps in an area of known occurrence in order to maximize the microhabitats sampled and increase the chance of collecting these enigmatic insects.

Conflict of Interests

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

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Research Article

Molecular Population Structure of *Junonia* Butterflies from French Guiana, Guadeloupe, and Martinique

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Up to 9 described species of *Junonia* butterflies occur in the Americas, but authorities disagree due to species similarities, geographical and seasonal variability, and possible hybridization. In dispute is whether Caribbean *Junonia* are conspecific with South American species. *Cytochrome oxidase I* (*COI*) barcodes, *wingless* (*wg*) sequences, and Randomly Amplified Fingerprints (RAF) were studied to reveal *Junonia* population structure in French Guiana, Guadeloupe, Martinique, and Argentina. Phylogenetic analysis of *COI* recovered 2 haplotype groups, but most *Junonia* species can have either haplotype, so *COI* barcodes are ambiguous. Analysis of nuclear *wingless* alleles revealed geographic patterns but did not identify *Junonia* species. Nuclear RAF genotyping distinguished 11 populations of *Junonia* arranged into 3 clusters. Gene flow occurs within clusters but is limited between clusters. One cluster included all Argentinian samples. Two clusters included samples from French Guiana, Martinique, and Guadeloupe and appear to be divided by larval host plant use (Lamiales versus Scrophulariales). Many *Junonia* taxa were distributed across populations, possibly reflecting patterns of genetic exchange. We had difficulty distinguishing between the Caribbean forms *J. zonalis* and *J. neildi*, but we demonstrate that Caribbean *Junonia* are genetically distinct from South American *J. evarete* and *J. genoveva*, supporting the taxonomic hypothesis that they are heterospecific.

1. Introduction

Buckeye butterflies, genus *Junonia* (Nymphalidae), are an important model system for experimental research in the Lepidoptera [1, 2]. *Junonia* species have been widely used to study the evolution and development of butterfly wing colour patterns [2–9]. Experimental tools to manipulate gene expression developed in *Junonia* are broadly applicable across the Lepidoptera [10–14]. *Junonia* has also been used in studies of insect endocrinology [15–17] and has been an important system for examining the evolution of larval host plant preference and tolerance to host plant toxins [18–21].

Junonia butterflies are found throughout the Old and New World tropics. In the Western Hemisphere, forms of *Junonia* occur from southern Canada to Tierra del Fuego [22–24] and have a complicated taxonomic history. In 1775, Cramer [25] identified and described two similar species of *Junonia*, *J. evarete* and *J. genoveva*, from Suriname, a Dutch colony on the north coast of South America. The species were described according to the standards of the time (without designated type specimens) and the descriptions were accompanied by hand-tinted plates that reproduced the colours from the original watercolour drawings of specimens of each form (republished in [26]).

In the 20th century, there was considerable disagreement in the scientific community about whether Cramer's two species were truly distinct [27, 28] or whether all of the specimens belonged to *J. evarete* [29–33]. This is a result of the geographical [29, 34] and seasonal [35] variability of *Junonia* and the fact that some *Junonia* forms closely resemble one another [36, 37]. In addition, different *Junonia* forms share identical karyotypes ($N = 31$) [26, 38] and are capable of hybridization and the production of fertile offspring [39–41], further complicating the process of assigning names to *Junonia* specimens. These features make applying the biological species concept [42], phylogenetic species concept [43], or morphospecies concept [44] very difficult in *Junonia*. Operationally, we use the isolation species

concept that defines species as systems of populations such that genetic exchange between these systems is limited or prevented by one or more reproductive isolating mechanisms [45, 46]. Identifying and understanding the reproductive isolating mechanisms operating in *Junonia* will be of great importance in clarifying *Junonia* taxonomy.

Authorities who favoured the two-species hypothesis in *Junonia* called the larger form *J. genoveva* and the smaller form *J. evarete*. In 1985, Turner and Parnell [26], using specimens from Jamaica and Florida, USA, verified the existence of two *Junonia* species in both regions. However, after consulting Cramer's hand-tinted plates and comparing them to specimens from Jamaica and Florida, Turner and Parnell [26] switched the names so the larger species was now *J. evarete* and the smaller species was *J. genoveva*. Neild [22], using specimens from Venezuela (geographically much closer to the type locality of Suriname), also confirmed the existence of two species. However, Neild [22], unsatisfied with Cramer's [25] published plates (copies of which differ from one another due to variation among the watercolourists who tinted them and differences in how the plates aged), consulted Cramer's original watercolours and *Junonia* specimens from many localities in South America. Using this reference material, Neild [22] reversed Turner and Parnell [26] so that the larger species was again *J. genoveva* and the smaller species was *J. evarete*. Neild [22] also designated new types for *J. evarete* and *J. genoveva* to facilitate future taxonomic work.

Recently, L. Brévignon and C. Brévignon [23, 47, 48] identified 5 *Junonia* species (*J. evarete*, *J. genoveva*, *J. wahlbergi*, *J. litoralis*, and *J. divaricata*) from French Guiana. This represents the most diverse assemblage of *Junonia* in the New World. There are two forms of *Junonia* known from the Caribbean Islands, "*zonalis*" and "*neildi*," which were initially recognized as subspecies of mainland *J. evarete* and *J. genoveva*, respectively [49] and later as two distinct species: *J. zonalis* and *J. neildi* [23]. In recognizing *J. neildi* and *J. zonalis* as distinct species, L. Brévignon and C. Brévignon [23] restricted the use of the species epithets *J. evarete* and *J. genoveva* to mainland Central and South American forms. If it were confirmed that the Caribbean forms are actually distinct species with respect to *Junonia* from the mainland, this would explain some of the widespread difficulty of assigning appropriate taxonomic names to specimens from Florida, Jamaica, and elsewhere in the West Indies. Finally, there are two additional *Junonia* species, *J. coenia* from North America and *J. vestina* from the Andes mountains of South America, for a current total of up to 9 species of New World *Junonia*.

The first molecular phylogenetic approaches to understanding the relationships among the species discussed here established that the New World fauna appears to be monophyletic and that the various New World forms are indeed in the genus *Junonia* [50, 51] (some authorities had previously placed these species in the related genus *Precis*) [52, 53]. Unfortunately, these early studies, which incorporated data from both mitochondrial and nuclear loci, had limited taxon sampling, including data from only 3 New World species [50, 51]. More recent studies of the molecular phylogeny of New World *Junonia* [23, 54, 55], which have better taxon sampling, have focused entirely on the mitochondrial *cytochrome oxidase I* (*COI*) locus, which is widely used as a barcoding locus for animal taxa [56, 57]. Based on mitochondrial haplotype sequences, the relationships among many New World *Junonia* species are ambiguous and most species are not reciprocally monophyletic [23, 24, 54]. The degree to which recent divergences, retained polymorphisms, and/or hybridization events contribute to these patterns in *Junonia* is unknown because only mitochondrial markers were considered. What is apparent is that there are two very divergent *COI* haplotype groups (4% sequence divergence between them) present in New World *Junonia*: Group A, which predominates in South America and is also present in the Caribbean, and Group B, which predominates in North and Central America but which also occurs in the Caribbean and South America [24, 54]. Sequences belonging to each of these haplotype groups can occur in different individuals of the same species at the same locality [24].

The most successful study to date to distinguish between New World *Junonia* taxa using molecular markers employed a combination of mitochondrial and nuclear markers to examine populations of *Junonia* in Buenos Aires, Argentina. Borchers and Marcus [24] used DNA sequences from the nuclear *wingless* gene and anonymous nuclear loci identified by Randomly Amplified Fingerprinting (RAF) (a technique used to assess genetic diversity within populations [58–60] and gene flow between populations [61]) in addition to sequences from the mitochondrial *COI* gene. They identified 3 distinct populations of *Junonia* from Buenos Aires: one population with dark-coloured wings referred to as *J. evarete flirtea* [62] which Borchers and Marcus [24] suggested may correspond to *J. wahlbergi* and 2 light-coloured populations that correspond to *J. genoveva hilaris* and either a genetically disparate population of *J. genoveva hilaris* or an undescribed cryptic *Junonia* species. However, the relationship of these Argentinian forms with *J. evarete* and *J. genoveva* from Suriname and French Guiana is not known, so we refer to them as *J. "flirtea"* and *J. "hilaris."*

In the current study, we extend the genetic tools that were employed by Borchers and Marcus [24] to *Junonia* populations from French Guiana and the French Antilles in order to study the distinctiveness of the named taxa within and between these two localities. This will allow an explicit test of the 7-species taxonomic hypothesis (2 species in the French Antilles plus 5 species in French Guiana) of L. Brévignon and C. Brévignon [23] and also detect possible hybridization events between named forms. By using a common set of markers we will also be able to compare these populations to previously studied *Junonia* from Argentina [24].

2. Materials and Methods

2.1. Specimens and DNA Preparation. A total of 104 *Junonia* specimens were collected from the wild as adults, reared from wild-collected larvae, or reared from eggs laid by wild-collected adults and frozen at −20°C (Table 1). DNA was isolated from legs removed from each specimen. Some samples (42 specimens) were prepared by the Canadian Centre for DNA Barcoding at the University of Guelph as previously described [23]. The remaining samples (62 specimens) were

Table 1: Number of *Junonia* specimens included in this study either entirely processed in our laboratory or extracted at the University of Guelph and sent to us for further study.

Species and locality	DNA extracted in our laboratory	DNA extracted by Guelph and whole genome amplified by our laboratory
J. coenia, Florida, USA	0	2
J. divaricata, French Guiana	0	5
J. evarete, French Guiana	0	6
J. genoveva, French Guiana	32	6
J. litoralis, French Guiana	8	4
J. neildi, Guadeloupe	6	2
J. neildi, Martinique	2	3
J. wahlbergi, French Guiana	0	10
J. zonalis, Guadeloupe	7	2
J. zonalis, Martinique	7	2

processed in our laboratory using the Qiagen DNEasy Blood and Tissue kit (Qiagen, Düsseldorf, Germany) as previously described [24], except that the extractions were performed in a Qiagen QIAcube instrument using the standard instrument protocol for purification of total DNA from animal tissue. Extracted DNA was stored at −20°C.

Only 10 μL aliquots of DNA were available for the 42 *Junonia* specimens processed at the University of Guelph, which was insufficient for the number of experiments we wished to conduct. To produce additional template, whole genome amplification using Illustra Genomiphi V2 (GE Health Care Life Sciences, Pittsburgh, PA, USA) protocol was performed as follows: 1 μL of DNA template and 9 μL of sample buffer were incubated at 95°C for 3 min, cooled to 4°C, mixed with 9 μL of reaction buffer and 1 μL of enzyme, incubated at 30°C for 90 minutes and then 65°C for 10 minutes, and cooled to 4°C. Deionized distilled water was used as the template for a Genomiphi amplification negative control. Genomiphied samples were stored at −20°C.

2.2. Mitochondrial Cytochrome Oxidase I Protocol. *Cytochrome oxidase I* (*COI*) PCR products were generated using a seminested two-step amplification with LCO1490 and Nancy primers followed by a reamplification with LCO1490 and HCO2198 (Table 2) [63, 64]. Quick-Load Taq 2X Mastermix (New England Biolabs, Ipswich, MA, USA) was used in PCR reactions with total volumes of 25 μL. Amplification protocols were run on a BioRad MyCycler or S1000 Thermal Cycler (BioRad, Hercules, California, USA) for these and all other PCR amplifications unless otherwise specified. LCO1490/Nancy PCR reaction conditions were 95°C for 5 minutes; 40 cycles of 95°C for 1 minute, 46°C for 1 minute, 72°C for 1.5 minutes; and a final 5-minute extension at 72°C before being placed on a 4°C hold. LCO1490/HCO2198 PCR reaction conditions were 95°C for 5 minutes; 35 cycles of 94°C for 1 minute, 46°C for 1 minute, 72°C for 1.5 minutes; and a final 5-minute extension at 72°C before being placed on a 4°C hold. PCR reactions were evaluated by gel electrophoresis (1% agarose in TAE buffer, 78 V for 1 hour, visualized with ethidium bromide).

Samples that failed to amplify with LCO1490 and HCO-2198 were reamplified with M13-uniminibarF1 (miniCOIF) and M13-uniminibarR1 (miniCOIR) (Table 2) [65]. MiniCOI PCR reaction conditions were 95°C for 2 minutes; 5 cycles of 95°C for 1 minute, 46°C for 1 minute, 72°C for 30 seconds; 35 cycles of 95°C for 1 minute, 53°C for 1 minute, 72°C for 30 seconds; and a final 5-minute extension at 72°C before being placed on a 4°C hold. Further reactions were carried out to obtain overlapping PCR products that could be assembled as contigs to obtain additional sequence data. Additional primers were designed to bind to invariant regions of the *Junonia COI* gene (miniCOIF2 and miniCOIR2 in one reaction and either miniCOIF3 and HCO2198 or miniCOIF2 and HCO2198 (Table 2) in a second reaction) to selectively amplify required sequences. Reaction conditions for these primers were the same as the miniCOI protocol described above.

2.3. Nuclear Wingless Protocol. *Wingless* PCR products were generated using lepwg1 and lepwg2 primers (Table 2) [66]. *Wingless* PCR reaction conditions were 94°C for 5 minutes; 40 cycles of 94°C for 1 minute, 46°C for 1 minute, 72°C for 2 minutes; and a final 10-minute extension at 72°C before being placed on a 4°C hold. While these primers typically work well in *Junonia* [24], the samples analyzed here failed to produce detectable products, likely due to poor preservation of nuclear DNA. These PCR reactions were used as the template for PCR reamplification with miniwgF and miniwgR (Table 2), which we designed to bracket the most informative interval of the *Junonia wingless* coding sequence (Table 3). Mini-*wingless* reaction conditions were 95°C for 5 minutes; 40 cycles of 95°C for 1 minute, 57°C for 1 minute, 72°C for 1 minutes; and a final 5-minute extension at 72°C before being placed on a 4°C hold.

2.4. Sequencing. Correctly sized PCR products were sequenced as previously described [24]. Products were sequenced in both directions, usually with the same primers that generated the products. When the miniwgR primer produced poor quality sequences samples were reamplified with miniwgF and T7-miniwgR and sequenced using T7 primer (Table 2). Sequencing reactions were analyzed on an ABI 3730xl automated sequencer and edited using Sequencher 4.6 software [67]. Sequences were trimmed to the appropriate size (Table 3) and aligned in CLUSTALW [68].

2.5. Randomly Amplified Fingerprinting Protocol. Randomly Amplified Fingerprinting (RAF) was used to gather a large multilocus data set [60]. Amplifications were carried out using single fluorescently labelled primers that act as both forward and reverse primers. A product is produced only if the primers bind in the correct orientation and close enough to one another for amplification. The 3 RAF primers, each covalently bound to a 6-FAM fluorescent molecule (Integrated DNA Technologies, Iowa City, Iowa, USA), used in these amplifications were RP2 (5′-/6-FAM/ATGAAGGGGTT-3′),

Table 2: Primer sequences used in *cytochrome oxidase I* (*COI*) and *wingless* (*wg*) PCR reactions.

Primer name	Sequence
COI	
Nancy	5′CCCGGTAAAATTAAAATATAAACTTC3′
LCO1490	5′GGTCAACAAATCATAAAGATATTGG3′
HCO2198	5′TAAACTTCAGGGTGACC AAAAAATCA3′
M13-uniminibarF1	5′GTAAAACGACGGCCAGTGGAAAATCATAATGAAGGCATGAGC3′
M13-uniminibarR1	5′GGAAACAGCTATGACCATGTCCACTAATCACAARGATATTGGTAC3′
miniCOIF2	5′ATACTATTGTTACAGCCTCATGC3′
miniCOIR2	5′TGTTGTAATAAAATTAATAGCTCC3′
miniCOIF3	5′CCCCACTTTCATCTAATATTGC3′
wg	
lepwg1	5′GARTGYAARTGYCAYGGYATGTCTGG3′
lepwg2	5′ACTNCGCRCACCATGGAATGTRCA3′
miniwgF	5′ATCGCGGGTCATGATGCCTAATACG3′
miniwgR	5′GTTCTTTTCGCAGAAACCCGGTGAAC3′
T7-miniwgR	5′TAATACGACTCACTATAGGGGTTCTTTTCGCAGAAACCCGGTGAAC3′

Table 3: Expected sequence length of trimmed PCR products (primers removed) for each primer pair.

Primer pair	Trimmed sequence length (base pairs)
LCO1490/Nancy	725
LCO1490/HCO2198	658
M13-uniminibarF1/M13-uniminibarR1	153
mCOIF2/mCOIR2	292
mCOIF2/HCO2198	520
mCOIF3/HCO2198	295
Lepwg1/Lepwg2	402
miniwgF/miniwgR	137

RP4 (5′-/6-FAM/TGCTGGTTCCC-3′), and RP6 (5′-/6-FAM/TGCTGGTTTCC-3′) [59]. Amplifications were performed in triplicate along with positive and negative (distilled deionized water) controls for a total of 954 RAF amplifications. Reaction volumes of 10 μL were used. Samples were run in a BioRad MyCycler Thermocycler under the following reaction conditions: 95°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 57°C for 1 minute, 56°C for 1 minute, 55°C for 1 minute, 54°C for 1 minute, 53°C for 1 minute; and a final 5-minute extension at 72°C before being placed on a 4°C hold. Reactions were shipped at room temperature to the Biotechnology Core Facility at Western Kentucky University (Bowling Green, Kentucky, USA). 10 μL HiDye formamide and 1 μL RX-500 GeneScan Size Standard (Applied Biosystems, Carlsbad, California, USA) were added to each PCR tube upon receipt. The solution was then vortexed for 1-2 seconds and placed in a microcentrifuge at 13,000 rpm for 30 seconds at room temperature. Samples were placed into individual wells on a sequencing plate and incubated at 95°C for 4 minutes in a thermocycler. Following 3–5 minutes on ice, samples were loaded into an ABI 3130 automated sequencer (Applied Biosystems), which was fitted with a 50 cm capillary filled with Pop-7 sequencing polymer for fragment analysis.

2.6. Mitochondrial Cytochrome Oxidase I Analysis. A subset of the samples in the current study was used in a previous barcoding study performed in another laboratory [23]. To ensure that there was no confusion or contamination of DNA samples during transfer we resequenced *COI* from 17 samples (of 42 transferred) that had been previously sequenced. In all cases, identical sequences were obtained by our laboratory as previously reported [23]. *COI* sequence alignments were converted to NEXUS format for phylogenetic analysis using several different reconstruction methods (distance, parsimony, and likelihood) that rely on vastly different assumptions about sequence evolution, each of which recovered essentially the same tree. For the sake of brevity, we will only present the maximum likelihood analysis (HKY model, 10 replicate heuristic searches with random number seeds, tree bisection, and reconnection branch swapping algorithm) [69]. Other previously published *Junonia COI* sequences were included in this phylogenetic analysis [23, 24, 51, 57, 70–74]. We also conducted a maximum likelihood bootstrap analysis of this dataset (500 fast addition replicates, collapsing all notes with frequency less than 50%). The aligned *COI* FASTA sequences generated by this study along with 22 previously published Argentinian *Junonia COI* sequences [24] were analyzed using Arlequin 3.5 [75]. We employed an AMOVA analysis with the following settings: 1000 permutations, determining the minimum spanning network (MSN) among haplotypes, computing distance matrix, and pair-wise difference with a gamma value of 0. The minimum spanning tree output from AMOVA was put into HapSTAR-0.7 [76], which displays the haplotype network in graphical form. Since analysis in Arlequin requires all sequences to be of the same

length, the analysis was first conducted using all samples that amplified using LCO1490/HCO2198 (Figure 2) and then repeated after trimming all sequences to the length of miniCOIF2/HCO2198 (Figure 3). Additional adjustments to the network were made using Canvas X (ACD Systems, Seattle, Washington, USA) such as scaling the population circles to reflect sample size and adding pie charts to reflect the RAF population assignment or geographical location and species.

2.7. Nuclear Wingless Analysis. For the *Junonia* species sequenced in this study and Argentinian *Junonia wingless* sequences from a prior study [24], individuals heterozygous for single nucleotide polymorphisms (SNPs) in the coding sequence were identified using sequencing chromatograms and CLUSTALW alignments. For each polymorphism, the genotype of each individual was entered into PHASE 2.1.1 [77] and analyzed using the default settings. PHASE uses the Markov Chain-Monte Carlo method to group coinherited SNPs in order to determine the most probable *wingless* alleles present in each individual. The most likely alleles identified in PHASE were assigned to each individual and the data was then entered into GENEPOP 4.0.10 [78]. GENEPOP was used to test for genetic differentiation (Exact G test [79]) by determining if the alleles from each subpopulation were drawn from the same distribution. GENEPOP settings used for testing all populations were a demorisation of 10,000, 10,000 batches and 10,000 iterations per batch. Finally, Structure 2.3.3 [80] was used to analyze the *wingless* data since, unlike GENEPOP [78], Structure does not require the *a priori* assignment of individuals to specific subpopulations. Population structure exhibited by *wingless* alleles was analyzed using Structure 2.3.3 [80] with settings for codominant alleles, a 10,000 step burn-in and 1 million Markov Chain-Monte Carlo Method replicates. Ten replicate structure searches tested each of 15 different population models with 1 to 15 subpopulations among the 88 *wingless* sequences. The maximum log likelihood ($\ln P(D)$) for the 10 replicate searches for each population model was used to calculate the posterior probability ($P(K = n)$) of each population model. Haplotype networks of *wingless* alleles were constructed in the same manner as *COI* except that PHASE output identifying the most likely *wingless* genotypes was formatted for input into Arlequin 3.5 [75].

2.8. Randomly Amplified Fingerprinting Analysis. Fragment analysis sample runs were combined with previously studied Argentinian *Junonia* [24] and analyzed using GENEMAPPER version 3.7 software (Applied Biosystems). An allelic bin size of 3 base pairs was selected in order to detect polymorphic alleles without introducing excessive noise into the analysis associated with small differences in run time between samples. The resulting GENEMAPPER genotypic classifications were exported to an Excel spreadsheet (Microsoft, Redmond, Washington, USA) for further analysis. Bands that appeared in negative control amplifications (of deionized distilled water with no DNA added) were considered artefacts and removed from further analysis for all samples. Within the 3 replicate RAF fragment runs for each primer from an individual butterfly, allele-calling for the presence or absence of the dominant allele at each RAF locus was based on a majority rule determination (at least 2 of the 3 runs had to show the allele for it to be scored as present). Each locus was coded in binary with 0 indicating the absence of an allele and 1 indicating the presence of the allele. Such binary data was analyzed using Structure 2.3.3 software [80] with the same settings as described previously for the *wingless* data except that in the case of the RAF data set only dominant alleles could be scored. A total of 50 replicate searches were carried out on each of K = 1–15 populations, first including only the samples genotyped in this study (primarily from French Guiana and the Caribbean) and then again including the 22 Argentinian specimens genotyped in a previous study [24].

Allele frequencies for each RAF locus were calculated for each population identified in Structure and formatted for input for the CONTML application of PHYLIP 3.5 [81] as implemented in EMBOSS Explorer [82]. CONTML uses a rigorous maximum likelihood algorithm to estimate phylogenies based on allele frequencies. In this model, all divergence between populations is assumed to be due to genetic drift in the absence of new mutations [83]. CONTML trees were exported in NEXUS format and rendered in EvolView [84] for interpretation. A parallel analysis was conducted in CONTML for the RAF data set and the allele frequency data obtained for *COI* and *wingless*.

3. Results

3.1. Mitochondrial Cytochrome Oxidase I Results. New *COI* DNA sequences generated by this project were deposited in Genbank (67 accessions, numbers KJ469059–KJ469126), with the exception of specimens with only *COI* minibarcode sequence fragments [65], which were submitted to the DNA Databank of Japan (DDBJ, 5 accessions AB9353-41–AB935345). Full *COI* barcode sequences, covering the interval between LCO1490 and HCO2198, were recovered from 65 specimens (17 reported previously [23] and 48 new sequences), 15 of which required assembling 3 sequence contigs to obtain the 658 bp sequence. Partial barcode sequences were obtained from miniCOIF/R sequences assembled into contigs with miniCOIF2/R2 sequences (2 specimens), miniCOIF/R sequences assembled into contigs with miniCOIF3/HCO2198 sequences (2 specimens), and miniCOIF2/HCO2198 sequences alone (16 specimens). Overall, some *COI* sequence was recovered from 90 of the 104 specimens.

Analysis of the *COI* sequences produced a maximum likelihood phylogenetic tree (Figure 1). As previously reported [24, 54], there are two distinct mitochondrial haplotype groups in New World *Junonia*. Haplotype group A is found in South American and Caribbean specimens, while haplotype group B includes many North American, Central American, and Caribbean specimens, as well as some South American specimens. A few forms of *Junonia* appear to be associated with only one haplotype group (group A: the South American forms *J. "flirtea"* and *J. vestina*; group B: the North American forms *J. coenia* and *J. "nigrosuffusa"*). All other *Junonia* species

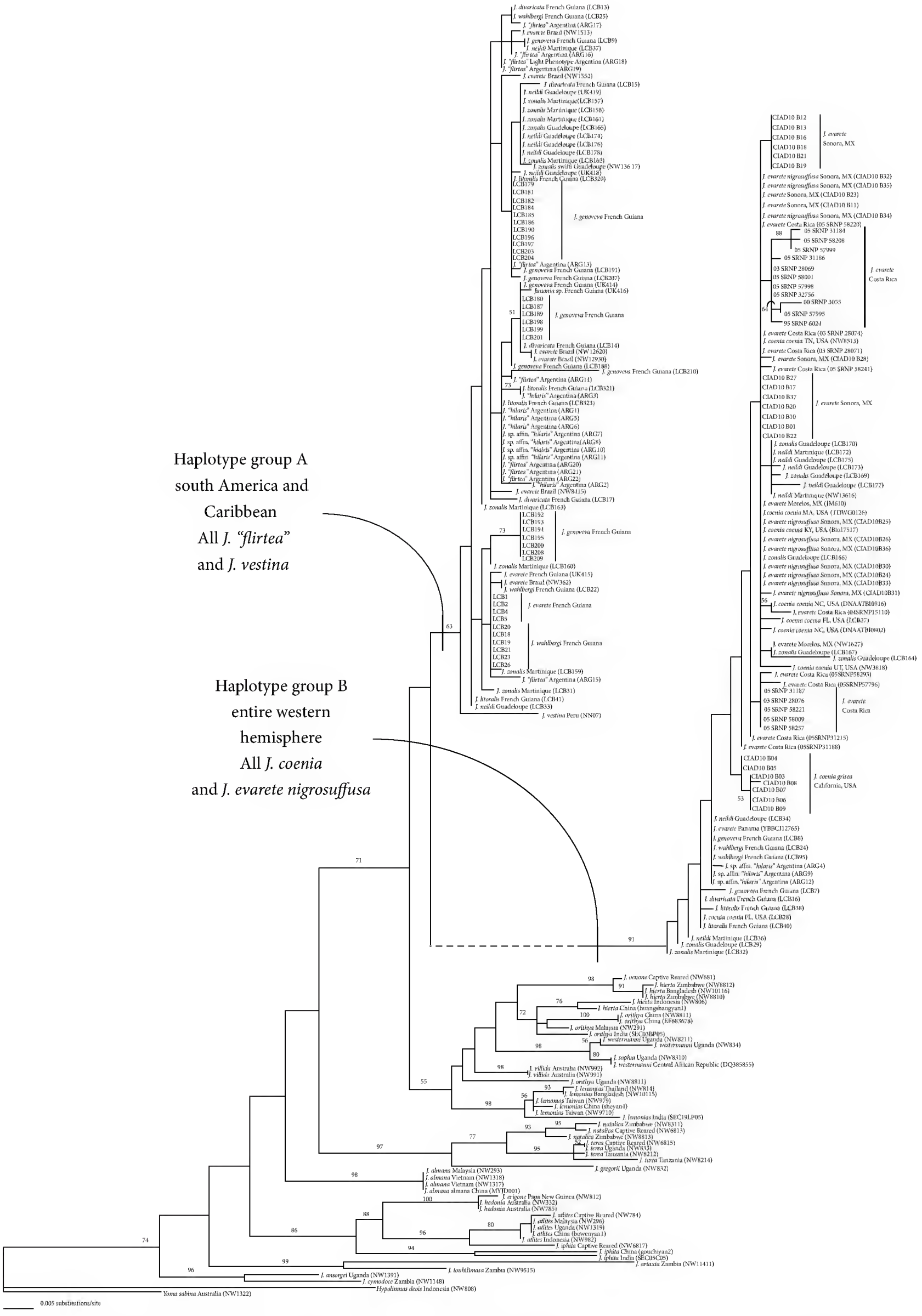


FIGURE 1: Phylogenetic tree depicting the two distinct mitochondrial *COI* haplotype groups found among New World *Junonia*. Group A haplotypes occur primarily in South America and the Caribbean. Group B haplotypes occur throughout the Western Hemisphere but are most common in North and Central America. Most *Junonia* species include individuals with both haplotypes. Only *J. "flirtea"* and *J. vestina* possess exclusively group A haplotypes, while only *J. coenia* and *J. evarete nigrosuffusa* possess exclusively group B haplotypes. In this figure, the entire haplotype Group B clade has been translated horizontally so that the tree fits better on the page. The dotted portion of the line connecting this clade to the tree is not included in calculations of sequence distances.

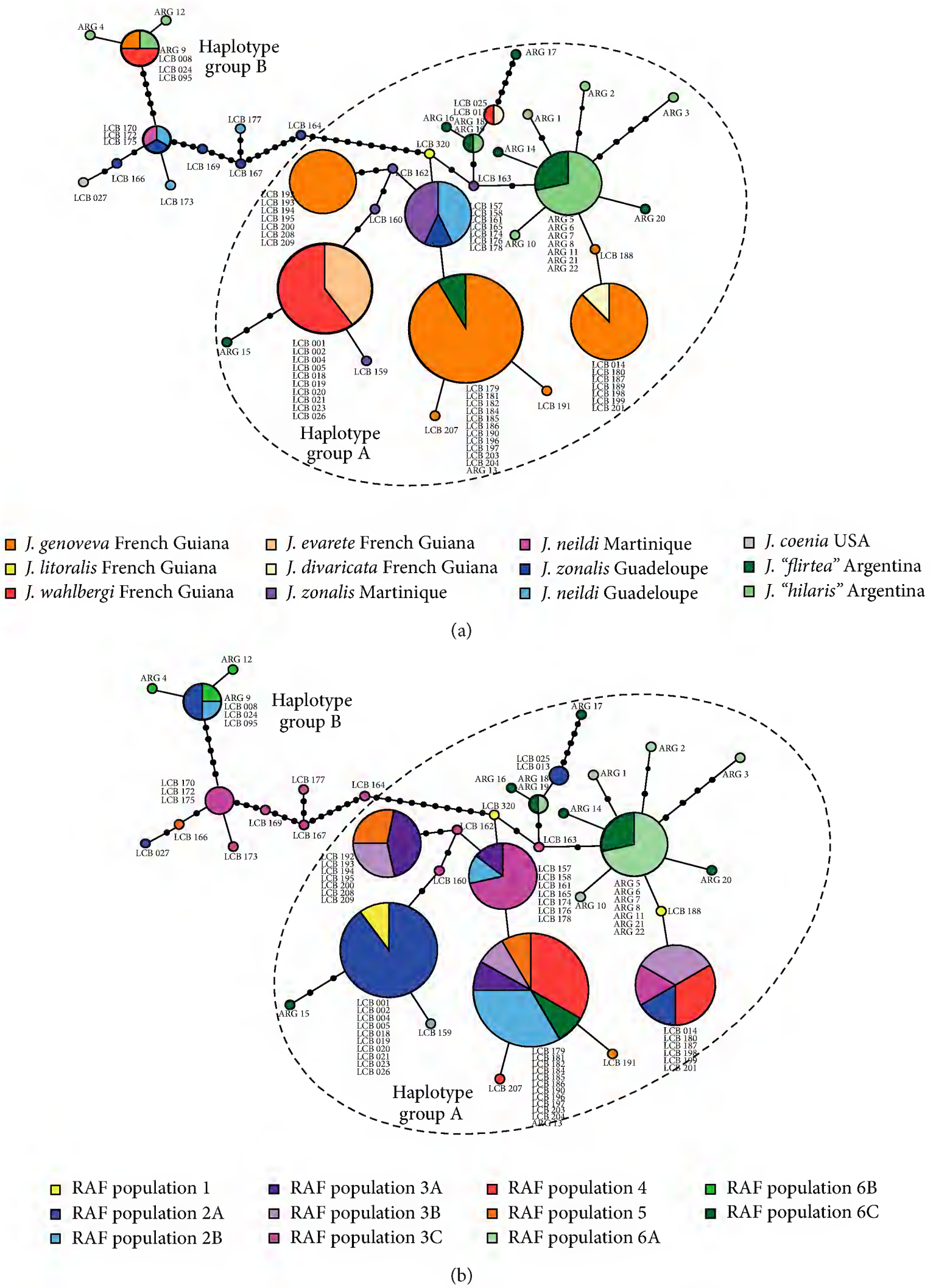


FIGURE 2: Haplotype networks generated using complete barcode fragment mitochondrial *COI* haplotypes. Circles are scaled to represent the number of individuals that contain a specific *COI* haplotype. Divisions and colours of circles in (a) reflect geography and species associated with each *COI* haplotype. French Guianan *J. genoveva* is orange, French Guianan *J. litoralis* is yellow, French Guianan *J. wahlbergi* is red, French Guianan *J. evarete* is pale orange, French Guianan *J. divaricata* is pale yellow, American *J. coenia* is grey, Martiniquan *J. zonalis* is purple, Martiniquan *J. neildi* is pink, Guadeloupean *J. zonalis* is dark blue, Guadeloupean *J. neildi* is light blue, Argentinian *J. "hilaris"* is light green, and Argentinian *J. "flirtea"* is dark green. Colours of circles in (b) reflect the RAF population associated with each *COI* haplotype. Population 1 is yellow, Population 2A is dark blue, Population 2B is light blue, Population 3A is dark purple, Population 3B is light purple, Population 3C is pink, Population 4 is red, Population 5 is orange, Population 6A is light green, Population 6B is medium green, and Population 6C is dark green. All 11 RAF populations are represented in group A haplotypes. Group B haplotypes are comprised of RAF Populations 2A, 2B, 3C, 5, and 6B.

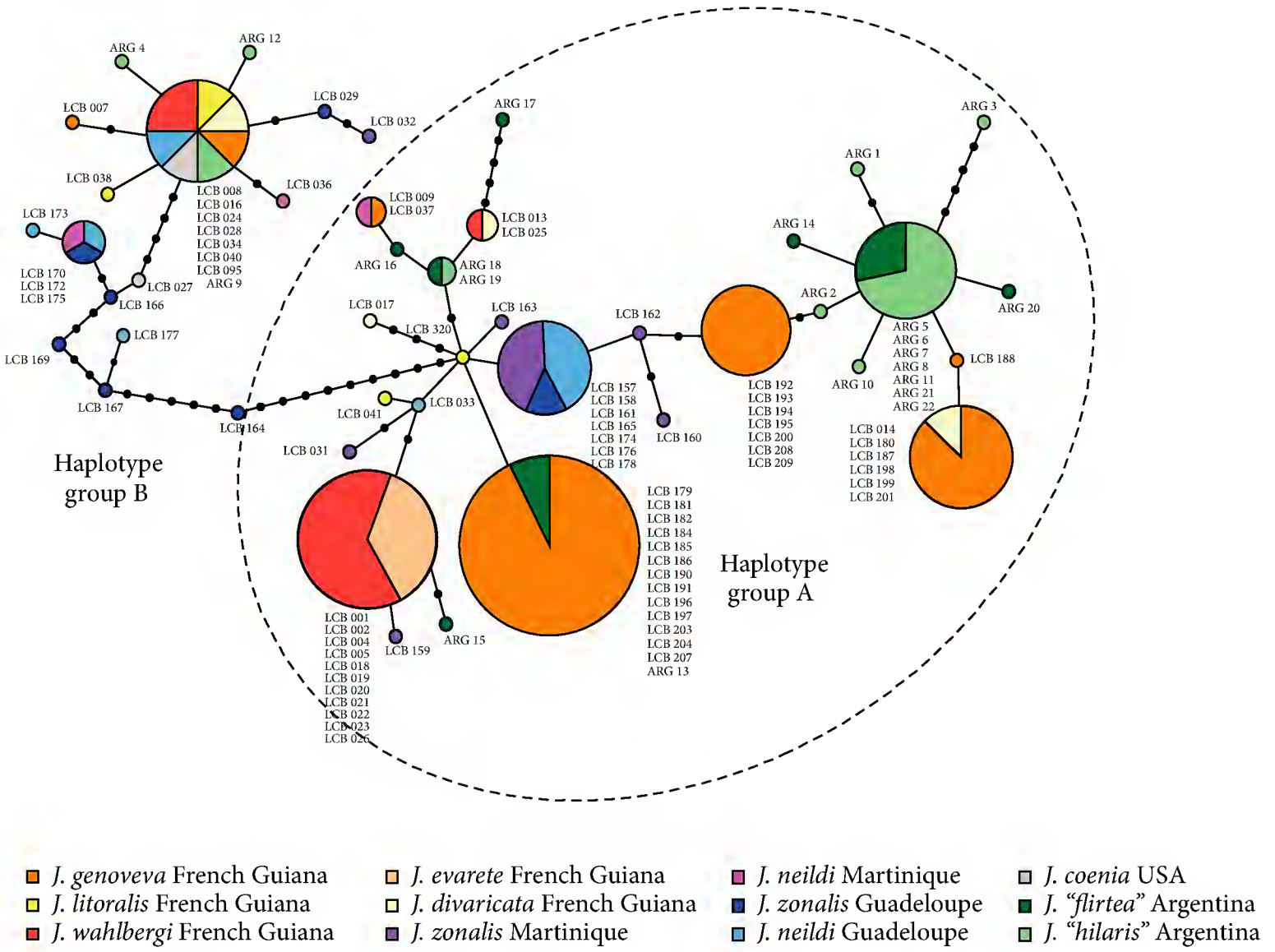


FIGURE 3: Haplotype networks constructed from those samples with partial mCOIF2/HCO fragment *COI* sequences. The network in (a) reflects geography and species while the network in (b) reflects RAF population assignment. The key to the colours is the same as in Figure 2.

(*J. divaricata*, *J. evarete*, *J. genoveva*, *J. "hilaris," J. litoralis*, *J. neildi*, *J. wahlbergi*, and *J. zonalis*) were found to include individuals with haplotypes in both group A and group B. *COI* coding sequences from the two haplotype groups contain no internal stop codons, no insertions or deletions, and few (and generally conservative) nonsynonymous substitutions and show little evidence of heterozygosity (no double bands in PCR products, very few double peaks in sequencing reads to indicate heterozygous sites within PCR products), suggesting that these are not pseudogenes or nuclear copies of mitochondrial DNAs, but true allelic alternatives.

Junonia litoralis from French Guiana and *J. neildi* from the Caribbean, both of which feed on black mangrove (*Avicennia germinans*) as larvae, include individuals with mitochondria from both *COI* haplotype groups. *Junonia genoveva* from French Guiana, which has often been considered conspecific with *J. neildi* from the Caribbean, also includes individuals that carry haplotypes in groups A and B. *Junonia evarete* from French Guiana have exclusively group A *COI* haplotypes, but *J. zonalis* from the Caribbean,which has sometimes been considered conspecific with *J. evarete*, includes individuals from both haplotype groups. In specimens from Guadeloupe, regardless of species, group A haplotypes are relatively rare (5/15, 33% type A). Specimens from Martinique, which is closer to the South American mainland than Guadeloupe, primarily have group A haplotypes (9/12, 75% type A). Specimens from French Guiana, regardless of species, primarily have group A haplotypes (49/56, 87.5% type A) as do specimens from Argentina (19/22, 86% type A).

Analysis of the haplotype networks produced from *COI* sequences revealed the same general pattern regardless of whether 86full-length 658 bp barcode sequences (Figure 2) or 102 partial 520 bp barcode sequences (Figure 3) were analyzed. Haplotype groups A and B are clearly delineated with 11 nucleotide changes between the genotypes from groups A (*J. zonalis* specimen LCB164) and B (*J. litoralis* specimen LCB320) that are most similar to each other. The two networks differ primarily with respect to how some group A genotypes are connected to LCB320, a specimen of *J. litoralis* located near the center of haplotype group A. There are also some minor rearrangements among the genotypes in haplotype group B between the two networks. There is a strong geographic signal in the *COI* haplotype network with specimens from the same place often sharing identical or similar genotypes. Many genotypes are found only in French Guiana, the French Antilles, or Argentina and there is only one group B genotype that can be found in all 3 localities (Figure 3(a)).

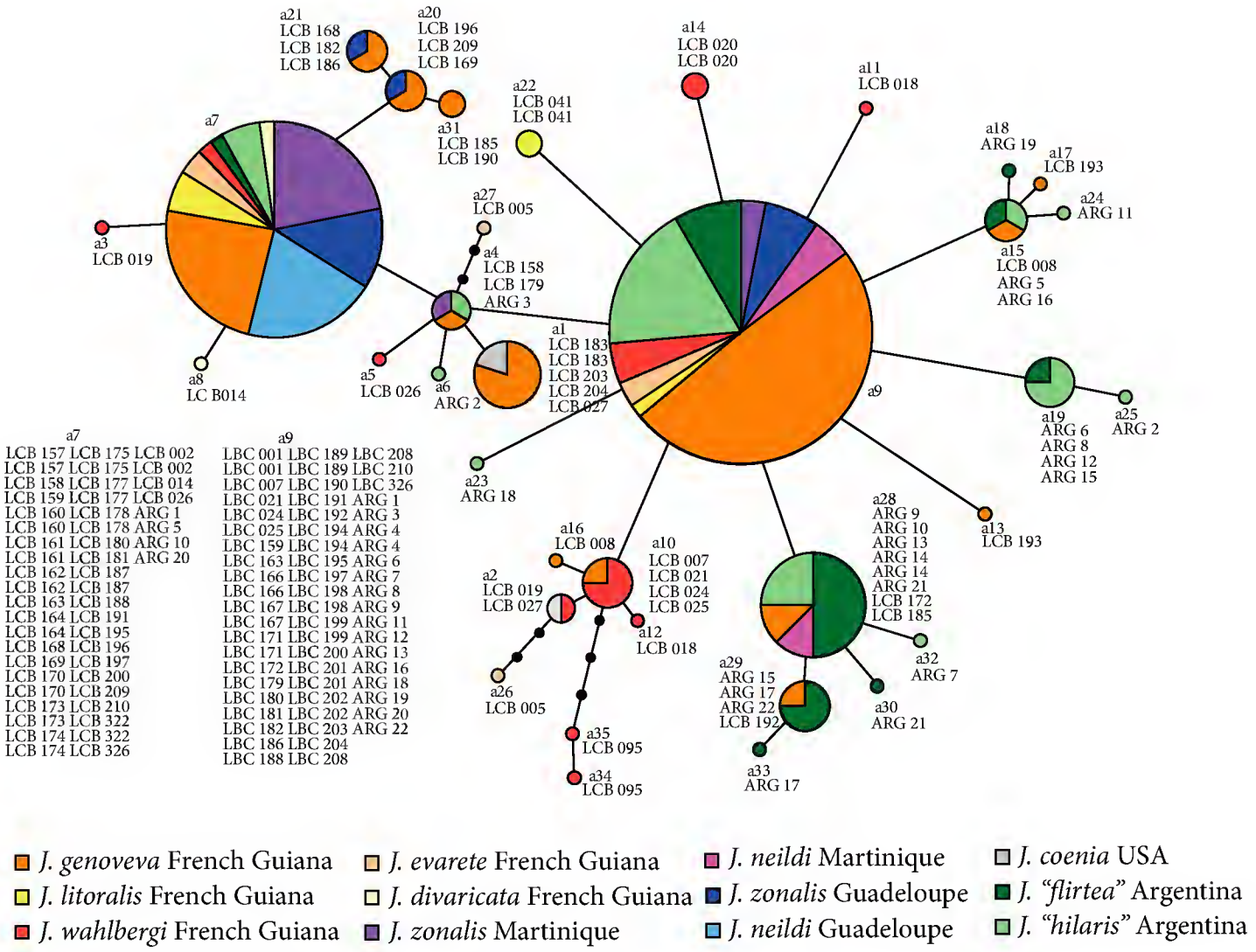
The haplotype networks also reveal that almost all *J. wahlbergi* and almost all *J. evarete* from French Guiana share a single group A genotype that is rare in other *Junonia* species. Similarly, the vast majority of *J. genoveva* from French Guiana possess one of 3 disparate genotypes within *COI* haplotype group A (Figures 2(a) and 3(a)) that are rare in all other *Junonia* species. Most of the remaining *J. genoveva* specimens have sequences that are one nucleotide removed from one of the three haplotype A genotypes or carry a genotype from haplotype group B. For most other species of *Junonia* there are no abundant *COI* genotypes that are diagnostic for particular species.

3.2. Nuclear Wingless Results. Short 137 bp *wingless* sequences were recovered from 66 of the 104 specimens. The newly generated sequences were submitted to DDBJ (accession numbers AB935346–AB935395 and AB936758–AB936773). These sequences were analyzed in combination with 22 *wingless* sequences from Argentinian *Junonia* specimens [24]. Following CLUSTALW alignments of the sequences of the mini-*wingless* PCR products with the Argentinian *wingless* products, 22 single nucleotide polymorphisms (SNPs) were identified in this highly variable region. These sites were confirmed in the chromatograms by the presence of a double peak, which indicates heterozygosity. Of the 22 SNPs, 21 are binary SNPs and 1 SNP contains 3 alternate nucleotides. Analysis of the SNPs in PHASE 2.1.1 [77] identified a total of 35 *wingless* alleles. The most probable allelic combinations for each *Junonia* specimen were identified in PHASE and then assigned to each individual for entry into GENEPOP 4.0.10 [78].

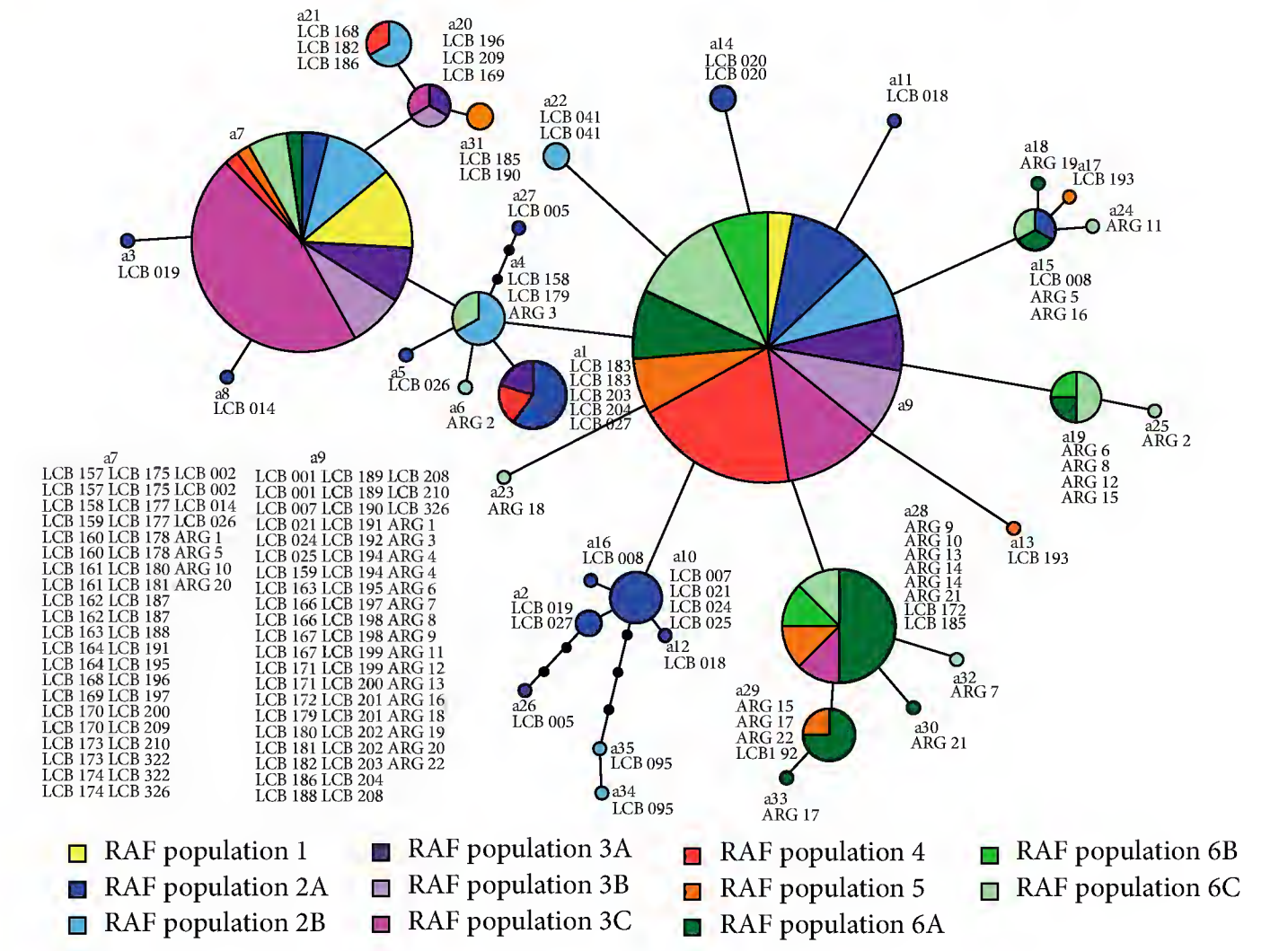
GENEPOP was first used to test for genetic differentiation among all populations. Separating *Junonia* populations solely by geography ($P = 0.00017$) or species ($P = 0.0000$) and by both geography and species ($P = 0.0000$) suggests significant genetic differentiation and distinct *wingless* allele distributions associated with these two factors. However, separating individual *Junonia* specimens using mitochondrial *COI* haplotype alone to define populations shows no statistically significant distinct distribution of *wingless* alleles associated with mitochondrial haplotypes ($P = 0.776$). However, when specimens were categorized by geography and *COI* haplotype ($P = 0.00014$); species and *COI* haplotype ($P = 0.0000$); or geography, species, and *COI* haplotype ($P = 0.0000$), the *wingless* alleles again appear to be drawn from significantly different distributions among subpopulations, likely due to the extremely strong influence of geography and species on the distribution of *wingless* alleles.

The *wingless* data was also analyzed in Structure 2.3.3 [80]. The results of the Structure analysis, testing for 1 to 15 subpopulations (Table 4), showed that the model with the highest posterior probability ($P(K = n)$) based on the *wingless* allelic data is $K = 1$ (all samples belonging to a single population). Haplotype networks of *wingless* alleles show that one allele (a9) that is common in *J. genoveva* populations from French Guiana (50%) is much rarer in *Junonia* from both Martinique and Guadeloupe (15%) (Figure 4). A second *Junonia wingless* allele (a7) is fairly common in both French Guianan (38%) and French Antillean (54%) populations.

3.3. Randomly Amplified Fingerprinting Results. RAF fragments for the RP2, RP4, and RP6 primers were recovered from all 104 *Junonia* specimens in this study and 22 Argentinian *Junonia* from our prior work [24]. RAF produces fragments of several different sizes from amplification with a given primer. 43 RP2 loci, 61 RP4 loci, and 18 RP6 loci were identified for a total of 122 variable RAF loci (Table 5). Structure 2.3.3 software [80] was used to test for 1 to 15 subpopulations among the French Guianan and Caribbean *Junonia* butterflies. The results of this analysis (Table 6) showed that the model with the highest posterior probability ($P(K = n) = 1$) is $K = 8$ (samples belonging to 8 separate populations).



(a)



(b)

FIGURE 4: Haplotype networks generated using *wingless* alleles. Circles are scaled to represent the number of individuals that contain a specific *wingless* allele with the exception of alleles a7 and a9 (if scaled proportionately, would be 50 and 61 times larger, resp., than shown). Colours for both (a) and (b) are as described in Figure 2. (a) Divisions and colours of circles reflect geography and species associated with each *wingless* allele. Allele a9 is much rarer in the Caribbean populations than it is in the mainland populations. 50% of a9 allele is comprised of *J. genoveva*. The majority of *wingless* alleles found in Argentinian *Junonia* are allele a9 or its derivatives. (b) Divisions and colours of circles reflect the RAF populations associated with each *wingless* allele. All 11 RAF populations carry the most common allele a9. Individuals from 10 of the 11 RAF populations carry the other common allele, a7.

Table 4: Inferring K, the number of populations, testing for 1–15 subpopulations in STRUCTURE, for Argentinian, French Guianan, Guadeloupean, and Martiniquan *Junonia wingless* data.

K (number of populations in model)	Median ln[$P(D)$]	PR ($K = n$) (posterior probability of the model)
1	−42.70	0.972
2	−46.54	0.021
3	−56.03	$1.587E-06$
4	−53.68	$1.664E-05$
5	−136.83	$1.287E-41$
6	−135.59	$4.426E-41$
7	−173.16	$2.136E-57$
8	−130.03	$1.156E-38$
9	−149.33	$4.797E-47$
10	−61.83	$4.806E-09$
11	−59.65	$4.251E-08$
12	−55.59	$2.465E-06$
13	−53.93	$1.296E-05$
14	−49.28	0.001
15	−47.82	0.006

Curiously, when this analysis was repeated with the inclusion of *Junonia* samples from Argentina, the model with the highest posterior probability ($P(K = n) = 1$) was $K = 6$. However, the Structure analysis did not distribute the Argentinian samples among the populations previously established for French Guiana and the Caribbean. Instead, all of the *Junonia* from Argentina were assigned to 1 population, while the samples from French Guiana and the French Antilles were redistributed among 5 populations. This was very curious because in our prior study the Argentinian *Junonia*, when analyzed by themselves using Structure, were divided into 3 populations [24]. The ability of Structure to detect population subdivision is reduced when sample sizes are very small, but the software is far more sensitive to insufficient numbers of variable markers [85]. This study employs more variable RAF markers (122 loci) than our earlier study (51 loci), but the additional loci are fixed in Argentinian *Junonia* [24]. When the Argentinian data set is analyzed in isolation both sets of. RAF loci show the model with the highest posterior probability ($P(K = n) = 1$) is $K = 3$ (samples belonging to 3 separate populations) as expected. Populations that were unchanged in composition whether or not Argentinian samples and were included in the analysis are populations 1, 4, and 5 (Figure 5). Structure identifies major discontinuities in population structure most readily. When both major and minor discontinuities exist in the same data set, the minor discontinuities can be missed because Structure employs a heuristic search algorithm that explores the solution space rather than calculating an exact solution [80]. In the absence of major discontinuities, the algorithm more readily identifies minor discontinuities. For populations that fused or divided between analyses with and without Argentinian samples, we reanalyzed each group of affected specimens in Structure separately from all other specimens and we used the population

Table 5: Fragment sizes, in base pairs, of RAF amplification products for RP2, RP4, and RP6 primer.

RP2 (bp)	RP4 (bp)	RP6 (bp)
35	37	35
39	42	38
41	46	47
43	50	50
47	56	61
49	58	63
51	62	72
54	65	73
56	68	77
58	71	82
60	79	85
64	82	89
66	85	93
68	87	147
72	89	149
75	93	190
76	95	193
79	99	197
80	100	
84	103	
86	112	
87	141	
88	145	
90	149	
92	152	
95	155	
98	158	
100	161	
107	164	
135	168	
136	175	
148	179	
150	196	
158	201	
160	202	
162	209	
182	223	
200	228	
228	231	
233	234	
234	237	
240	240	
241	247	
	249	
	258	
	261	
	277	
	280	
	287	
	289	

Table 5: Continued.

RP2 (bp)	RP4 (bp)	RP6 (bp)
	291	
	342	
	345	
	363	
	369	
	371	
	389	
	391	
	394	
	440	
	483	

assignments from these separate analyses as the definitive group definitions. Populations defined in this way are indicated by a shared number followed by letters (e.g., 2A and 2B).

Overall, there are 11 populations established by Structure analysis of RAF alleles (Figure 5(a)). RAF Population 1 (yellow) primarily includes specimens of *J. litoralis*, but 2 *J. zonalis*, 1 *J. genoveva*, and 1 *J. evarete* specimens also show genetic influence from this population.Population 2A (dark blue) includes all specimens of *J. wahlbergi* and *J. divaricata* and all but one of the *J. evarete* specimens. Population 2A also includes individuals from all of the other French Guianan and French Antillean *Junonia* species. Most of the specimens in Population 2B (light blue) also show genetic influence from Population 2A, but there are 2 Caribbean *J. zonalis* specimens whose primary genetic influence is population 2B. Population 3A (dark purple) is comprised of *J. genoveva* and *J. zonalis* specimens. Populations 3B (light purple) and 3C (pink) both include *J. genoveva*, *J. neildi*, and *J. zonalis* specimens. Population 4 (red) contains only *J. genoveva* specimens. Population 5 (orange) is primarily composed of *J. genoveva* specimens, although there is 1 *J. zonalis* specimen that also shows influence from this population. Populations 6A-C are the same as the 3 Argentinian *Junonia* populations previously described [24].

Finally, of particular interest is the distribution of specimens showing the genetic influence of more than one population. The populations exist in 3 distinct clusters with some genetic exchange within each cluster, but little apparent genetic exchange between clusters (Figure 5(a)). Populations 1, 3A, 3B, 3C, 4, and 5 belong to one such cluster with Population 3C as the "hub" population, showing genetic exchange with all of the other "satellite" populations, which have varying amounts of (often very limited) genetic exchange with one another. It is interesting to note that the 3C hub population is almost exclusively composed of specimens from the French Antilles. Specimens of *J. zonalis* and *J. neildi* from the Carib-bean and *J. genoveva* and *J. litoralis* from French Guiana comprise the remainder of this cluster (along with 1 specimen of *J. evarete*). In this cluster, many individuals have RAF genotypes with influence from more than 1 population, suggesting possible past or current genetic exchange among these populations. A second cluster includes Populations 2A and 2B, which show extensive genetic exchange between them, but limited genetic exchange with all of the other populations we have identified. The 2A-2B cluster shows a strong genetic influence from *J. evarete*, *J. wahlbergi*, and *J. divaricata* from French Guiana, although it also includes specimens from the other 2 French Guianan species, specimens of *J. neildi* and *J. zonalis* from the Caribbean, and *J. coenia* from Florida. A third cluster includes Populations 6A, 6B, and 6C and contains only Argentinian specimens. Like the 1-3A-3B-3C-4-5 cluster, one population (6B) has genetic exchange with the other two populations, but there may be little or no direct gene flow between 6A and 6C [24].

Table 6: Inferring K, the number of populations, testing for 1–15 subpopulations in STRUCTURE, for the Argentinian, French Guianan, Guadeloupean, and Martiniquan *Junonia* RAF data.

K (number of populations in model)	Median ln[$P(D)$]	PR ($K = n$) (posterior probability of the model)
Without Argentinian *Junonia*		
1	−235.98	9.598E − 47
2	−178.72	7.078E − 22
3	−147.20	3.458E − 08
4	−143.08	2.129E − 06
5	−136.83	0.001
6	−135.59	0.004
7	−173.16	1.839E − 19
8	−130.03	0.995
9	−149.33	4.130E − 09
10	−152.54	1.659E − 10
11	−146.13	1.008E − 07
12	−160.62	5.136E − 14
13	−150.26	1.630E − 09
14	−157.84	8.279E − 13
15	−157.205	1.562E − 12
With Argentinian *Junonia*		
1	−384.19	3.286E − 78
2	−300.45	7.665E − 42
3	−237.34	1.963E − 14
4	−208.28	0.082
5	−207.97	0.112
6	−206.27	0.611
7	−214.73	0.000
8	−208.28	0.082
9	−299.36	2.291E − 41
10	−313.86	1.156E − 47
11	−342.71	3.398E − 60
12	−258.91	8.416E − 24
13	−207.97	0.112
14	−243.61	3.714E − 17
15	−270.82	5.658E − 29

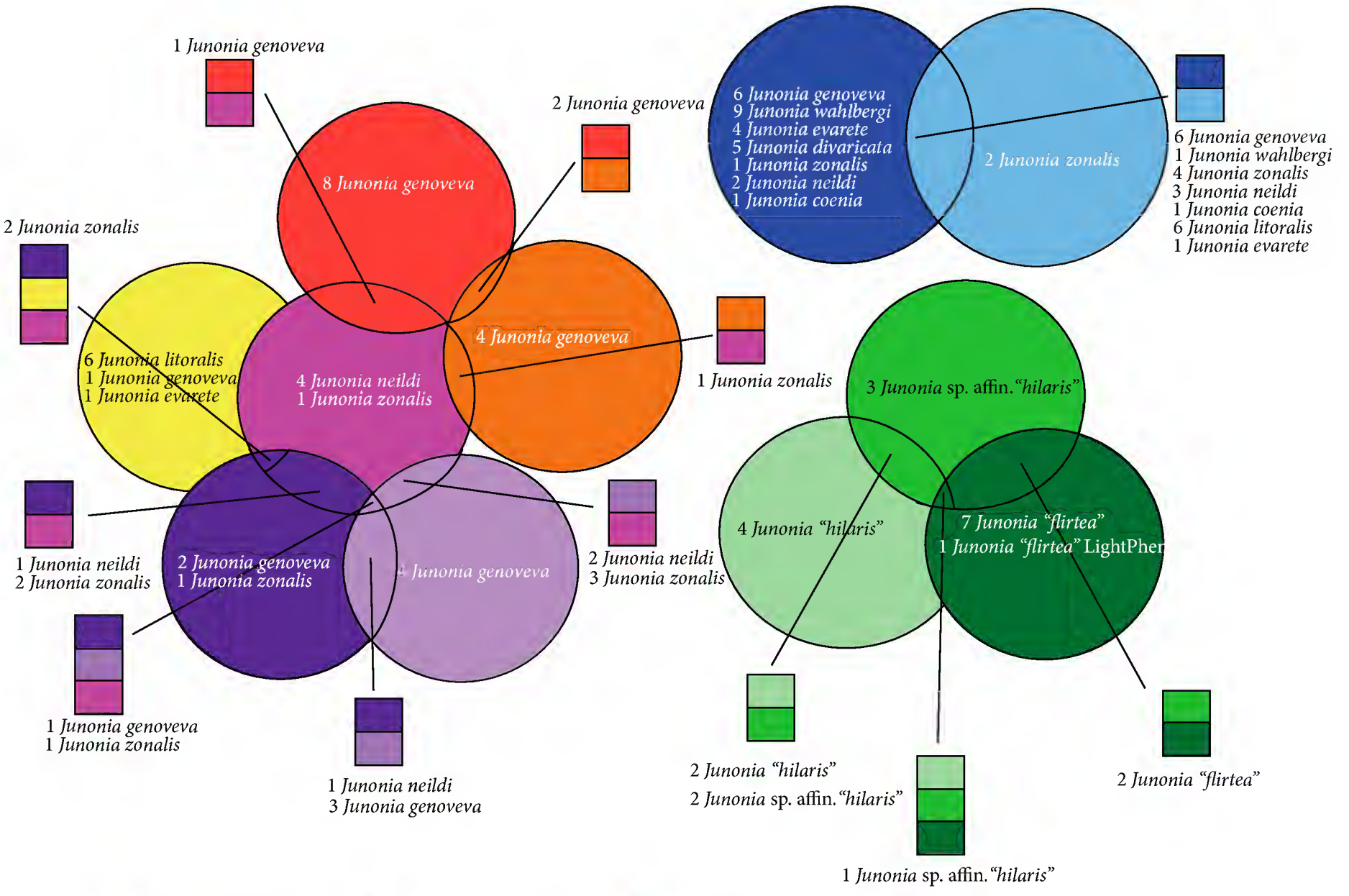


FIGURE 5: Summary of population genetic analysis of RAF nuclear genotyping. (a) Venn diagram illustrating gene flow occurring between populations. The 11 *Junonia* populations are distributed into 3 clusters which show extensive genetic exchange within a cluster, but little or no genetic exchange between clusters. (b) CONTML analysis of the RAF allele frequency data for populations identified in Structure. Clusters identified in (a) tend to be adjacent to one another on the tree, which shows a very strong influence on larval host plant use. Most of the individuals in RAF Populations 2A, 2B, 6A, 6B, and 6C are from species that utilize larval host plants in the order Scrophulariales. Virtually all of the *Junonia* in the remaining RAF populations are from species that use larval host plants in the order Lamiales.

CONTML analysis of the RAF allele frequency data for populations identified in Structure produced an arbitrarily rooted tree that shows much of the same clustering that was apparent from other analyses of this data set (Figure 5(b)). Populations 2A and 2B, which contain many Caribbean specimens, are sister groups on the tree while Populations 6A, 6B, and 6C, which consist of only Argentinian specimens, arise as an unresolved polytomy from a single common ancestor. The remaining populations, which make up the 1-3A-3B-3C-4-5 cluster, are also adjacent to one another on the CONTML tree, although they do not form a monophyletic group. CONTML analysis of the RAF allele frequency data combined with *COI* and *wingless* allele frequency data produces a tree of identical topology with minuscule changes in branch length (not shown).

4. Discussion

We hoped that using a set of molecular tools that had worked well to distinguish between forms of *Junonia* from Argentina [24] would allow us to unambiguously distinguish between forms from the French Antilles and French Guiana. Unfortunately, this is not entirely the case. *Cytochrome oxidase I* (*COI*) barcodes are clearly unreliable for distinguishing among most New World forms of *Junonia* because individuals of most named species contain haplotypes from either of the two main haplotype groups (A and B, Figure 1). This is consistent with the findings of prior studies that have found to be identical haplotypes in *Junonia* [23, 24, 54]. Certain *COI* haplotypes are found primarily in particular geographic regions, but most are found in more than one species in that region and, thus, are not diagnostic (Figures 2 and 3). There are examples of significant apparently intraspecific mitochondrial haplotype divergence in other species and are typically associated either with the existence of cryptic species (each with different haplotypes) [86, 87] or with hybridization followed by introgression of mitochondria [88, 89]. The presence of cryptic species is usually corroborated by the demonstration of either consistent phenotypic differentiation between cryptospecies, population subdivision based on nuclear markers between cryptospecies, or both. Neither this study nor previous studies of *Junonia* have been able to provide corroboration supporting the existence of cryptic species associated with *COI* haplotype diversity [23, 24, 54], so we feel weight of the evidence is more consistent with a history of hybridization and introgression. Except for certain special cases in which hybridization between forms of *Junonia* is apparently absent from a region [55], *COI* barcoding should probably be abandoned as a method for distinguishing taxa among New World *Junonia*. At the same time, the segregation of haplotypes A and B, separated by approximately 4% sequence divergence, in *Junonia* populations across much of the Western Hemisphere is a phenomenon worth studying in its own right.

There appears to be a north-south gradient across the Caribbean with respect to the frequency of *COI* haplotypes with the type A haplotype ranging from 0% in North America [54] to 87% in the South American mainland (this study and [24]). There is a long-standing hypothesis that Caribbean *Junonia* are a ring species [29, 34], which is defined as a group of species or subspecies that exhibit a ring-like distribution such that the forms at the extreme ends of the range overlap [42]. Gene flow occurs through intermediate forms in the middle of the ring, but, in a classic ring species, forms found in the overlapping region of the ring do not interbreed [90, 91]. Western Cuba and the nearby Island of Pines were identified as the possible region of overlap for the putative *Junonia* ring species since several phenotypically distinct forms of *Junonia* coexist there [34, 92]. Our results suggest that if there is a region of secondary contact between two ends of a Caribbean *Junonia* ring species, then hybridization between the terminal forms appears to be occurring and the zone of overlap is not limited to Cuba. More extensive mapping of the distribution of the haplotypes in combination with phylogenetic analysis of additional mitochondrial sequence data may provide insights into the origin of these haplotypes (currently unknown [51]) and what evolutionary forces may be contributing to their continued presence in *Junonia* populations.

Alleles from the nuclear locus *wingless*, which were helpful in distinguishing between *Junonia* from Argentina [24], were less effective in this study. This is probably due, at least in part, to the small 137 bp sequence fragment of *wingless* that we were able to recover from Caribbean and French Guianan *Junonia* (versus 402 bp previously recovered for Argentinian *Junonia* [24]). While the *wingless* fragment that was recovered is the most variable portion of the New World *Junonia wingless* coding sequence (Figure 4), potentially informative sequence variation elsewhere in the gene was not available for us to study. It would be highly desirable to obtain additional specimens of *Junonia* from French Guiana and the Caribbean with better-preserved nuclear DNA so that larger portions of the *wingless* gene could be analyzed. Another factor that may make *wingless* sequences less useful in French Guiana and the Caribbean is the large number of forms of *Junonia* coexisting and possibly interbreeding in this region [23]. If *wingless* coding sequences or sequences closely linked to *wingless* are adaptive, for example, contributing to colour pattern phenotypes under selection [8, 93], such sequences may be subject to introgression after hybridization events [94, 95]. As such, the evolutionary history of the introgressed region of the genome may not be representative of the evolutionary history of the organism as a whole [96]. Finally, *wingless* signalling may contribute to the development of colour pattern phenotypes that are used as field marks for identifying species of *Junonia* [8], most notably the prominent pale median stripe on the ventral hind wing of some species [97, 98]. This connection between developmental processes and species-specific phenotypes in combination with transgenic techniques [10–12, 14] may permit us to identify the specific mutations responsible for phenotypic evolution in *Junonia* and to characterize the molecular mechanisms responsible for colour pattern diversity.

In the past, Randomly Amplified Fingerprint (RAF) genotyping was extremely effective at distinguishing between forms of *Junonia* from Argentina [24]. In this study, these populations remained distinguishable from one another (Populations 6A, 6B, and 6C) and were genetically distinct from populations sampled from other regions (Figure 5). In fact, the Argentinian populations are all more genetically

similar to one another than to any populations in French Guiana or the French Antilles. Based on wing colour patterns, Argentinian *Junonia* have been conventionally referred to as *J. genoveva hilaris* (C. & R. Felder), the light buckeye butterfly, and *J. evarete flirtea* (Fabricius), the dark buckeye butterfly [62]. However, Borchers and Marcus [24] identified 3 genetically distinct Argentinian populations based on RAF genotypes. After consulting a key for the *Junonia* of French Guiana that relies on colour patterns and morphological traits [23], Borchers and Marcus [24] suggested that the two light-coloured Argentinian populations correspond to *J. genoveva* and either a genetically disparate population of the same species or an undescribed cryptic species while the dark-coloured population corresponds to *J. wahlbergi*. The results of this study suggest that there is no close affinity between the Argentinian forms and *J. genoveva* or *J. wahlbergi* from French Guiana, so the tentatively assigned scientific names should be revised. We refer to the 2 light-coloured forms as *J. "hilaris"* and *J.* sp. affin *"hilaris"* and the dark-coloured form as *J. "flirtea."*

The samples from French Guiana and the Caribbean are divided into two major clusters (Figure 5). The first cluster is composed of RAF Populations 1, 3A, 3B, 3C, 4, and 5. Overall, this cluster appears to be strongly associated with forms of *Junonia* whose larval host plants are in order Lamiales: *J. litoralis* from French Guiana and *J. neildi* from the Caribbean (both use larval host black mangrove, *Avicennia germinans*), *J. genoveva* from French Guiana (larval host *Hyptis atrorubens*), and *J. zonalis* from the Caribbean (larval hosts *Stachytarpheta jamaicensis*, *S. urticifolia*, and *Lippia nodiflora*) [23, 99]. Since all *Junonia* from the Caribbean feed on plants in the Lamiales, this cluster contains the bulk (19/30) of Caribbean specimens in our analysis. In contrast, the population cluster that includes Populations 2A and 2B contains the bulk of specimens from species whose larval host plants are in the order Scrophulariales: *J. coenia*, *J. divaricata*, *J. evarete*, and *J. wahlbergi*. *Junonia divaricata* and *J. evarete* use *Utricularia hispida* as their larval host, while *J. wahlbergi* and *J. evarete* use *Agalinis hispidula* [23]. *Junonia coenia* from North America feeds on a wide variety of larval hosts in the order Scrophulariales including several *Agalinis* species [18]. Sharing larval host plants may facilitate habitat overlap among extant forms and the cooccurrence of organisms is a necessary precondition for interspecific hybridization and gene flow to take place. The overall congruency between the population clustering based on RAF genotyping and host plant use supports the hypothesis of L. Brévignon and C. Brévignon [23] that host plant use defines two major lineages within New World *Junonia*. However, it should also be noted that there are exceptions to this congruence: 1 specimen of *J. evarete* clustered with the Lamiales feeders while 12 specimens of *J. genoveva*, 5 specimens of *J. zonalis*, and 5 specimens of *J. neildi* clustered with the Scrophulariales feeders (Figure 5). This might be interpreted as evidence for some gene flow between these two lineages.

Some species of *Junonia* were not readily distinguishable from one another in the Structure analysis of the RAF data. *Junonia coenia*, *J. divaricata*, *J. evarete*, and *J. wahlbergi* are all associated with the same RAF population cluster (2A-2B). We suspect that our inability to distinguish between these forms is due, at least in part, to artefact because these four species were represented by the smallest number of individuals in the RAF Structure analysis (between 2 and 10 individuals sampled depending on the species, Table 1). Thus, the statistical power of the algorithm is poor for these species [80]. With additional sampling of these forms a more robust analysis of population structure for these *Junonia* species will be possible. While we were not able to reliably separate *J. zonalis* and *J. neildi* from each other, we are able to conclude, based on the available data, that these Caribbean formsappear to be genetically differentiated from the taxa that occur in French Guiana with the vast majority of Caribbean specimens assigned to Populations 2B and 3C (Figure 5). This supports the taxonomic hypothesis of L. Brévignon and C. Brévignon [23], which elevated these taxa to full species. It also explains why it has been so challenging to apply taxonomic names based on South American types to forms found in the Caribbean [22, 26–28].

The other pattern emerging from the analysis of RAF genotypes is for individuals of one species to be spread across multiple RAF populations (Figure 5). *Junonia litoralis* from French Guiana has several individuals assigned to the same RAF population (Population 1), but that population also includes individuals from at least two other species. Furthermore, other *J. litoralis* are assigned to a different population cluster (Populations 2A and 2B). In the most extreme case of distribution among RAF populations, *J. genoveva* from French Guiana has individuals assigned to 6 RAF populations (spread across two major population clusters with apparently little gene flow between the clusters). *J. zonalis* from the French Antilles is similarly distributed across 4 populations while *J. neildi* is assigned to at least 2 populations.

This is similar to what has been observed previously for the light buckeye, *Junonia "hilaris"* from Argentina [24] and has been replicated in the current analysis of RAF data, which divided this species into two separate populations (Populations 6A and 6B). Previously, we suggested that the presence of two very phenotypically similar, but genetically distinct, populations of *Junonia* existing simultaneously in Buenos Aires, Argentina, may be due to mass migrations of individuals from geographically disparate areas [24]. Mass migration of *Junonia* is a phenomenon that has been documented in Argentina and elsewhere in the New World [100, 101]. However, mass migration of *Junonia* has not been observed in French Guiana (C. Brévignon, *pers. com.*) and mass migration followed by hybridization between forms would tend to homogenize the genotypes of the interacting populations over time. While some of the named *Junonia* taxa from French Guiana and the French Antilles have been described relatively recently [23, 47–49], all of these forms have been observed in the region for decades (C. Brévignon, *pers. com.*) and in some cases for centuries [25, 102].

Many different forms of New World *Junonia* tested in lab crosses are interfertile and produce viable fertile hybrids [39–41], but many of these interfertile forms are separated geographically or by habitat preference (see below) and would have limited contact in the wild. Preliminary attempts at some interspecific pairings of sympatric *Junonia* from French

Guiana have been unsuccessful (C. Brévignon, *pers. com.*). *Wolbachia* bacterial infections can prevent otherwise genetically compatible insects from producing viable offspring, blocking gene flow [103], perhaps contributing to the pattern we see in *Junonia*. Several species of *Junonia* have been tested for *Wolbachia* (including *J. evarete* from Panama), but thus far infections have only been detected in Asian *J. almana* [104, 105]. *Junonia* species have characteristic male genitalia [23, 24], but there is no evidence of male-female genitalic incompatibility as has been reported in some pairs of snail and moth sister species [106, 107]. Also, *Junonia* species have been observed to engage in courtship flights with heterospecifics in the wild [108] and wild-caught individuals that appear to be of hybrid origin have been identified based on their RAF genotypes [24] (Figure 5). This suggests that if mass migration were sufficiently common to bring individuals from geographically distinct populations into contact at a specific site, it would very quickly eliminate most of the genetic population structure in *Junonia* at that locality unless there is assortative mating between forms.

In addition to explaining patterns of genetic overlap among *Junonia* RAF populations, we have to explain the possible subdivision of some *Junonia* species across multiple populations (e.g., *J. genoveva* in French Guiana with 6 different RAF subpopulations, Figure 5). Frequently, individuals from one named taxon collected from a single locality were assigned to different RAF subpopulations. An alternative cause of the complex genetic population structure found in some forms of *Junonia* is that these species, which are defined on the basis of morphology and colour patterns, may include races that are specializing on different larval host plants. Host plant specialization is a widespread mechanism for population differentiation causing rapid evolution of adaptive traits for feeding on new hosts and for assortative mating to maintain favourable combinations of traits [109–113]. In some cases, it has been suggested that this has been a driver for reproductive isolation and incipient speciation in many insects [113–117].

Most New World *Junonia* are currently known to only feed on a single species of larval host plant in the wild [23, 49, 97]. However, *J. coenia* feeds on many alternative hosts [18]. Under artificial conditions, many varieties of *Junonia* larvae can be reared on alternate host plants or on artificial diets containing alternate host plant leaves ([40, 118] and Jeffrey M. Marcus, *pers. observation*). When presented with several alternative hosts, female *J. coenia* choose to oviposit on the same primary host plant used by the wild population from which the female was derived [21, 119]. If additional larval host plants for South American forms of *Junonia* exist, this may explain some of the extensive population structure seen in some species such as *J. genoveva*. New World *Junonia* host plants contain iridoid glycoside secondary compounds, the presence of which may be a necessary precondition for use as a host by *Junonia* [120]. This may help identify possible additional larval host plants for South American forms of *Junonia*.

A variety of mechanisms permit assortative mating and allow genetically distinct but reproductively compatible populations of species to persist in the same habitat. Habitat partitioning allows individuals from different populations to use different portions of available habitat, thereby making it less likely that they will interact and mate [121, 122]. In some North American habitats where multiple *Junonia* taxa occur, one of the authors (Jeffrey M. Marcus) has observed differences in habitat use. In mangrove swamps along the coast of Florida, USA, most *J. coenia* males appear to patrol mating territories in clearings without trees or other vertical habitat structure. In contrast, males of a form that resembles *J. neildi* (the forms have yet to be compared genetically) and whose larvae feed on black mangrove trees (*Avicennia germinans*) tolerate more vertical structure and establish mating territories in close proximity to their larval host plants. Similarly, in coastal dune habitats in south Texas, USA, *J. coenia* males establish mating territories on the foreshore between the sand dunes and the water line. A few meters away, males of the darkly pigmented *J. "nigrosuffusa"* (taxonomic affinities uncertain) appear to establish mating territories in the interdune and slack areas between the sand dunes. It is not clear whether these North American *Junonia* habitat preferences are due to preferences for abiotic conditions of the microhabitat itself or whether the presence or relative abundance of preferred larval host plants for each form in the favoured microhabitats is the driver of habitat preference [123]. In French Guiana, the presence of different *Junonia* taxa appears to be closely tied to the abundance and phenology of larval host plants [23]. Whether there are similar patterns of microhabitat subdivision among cooccurring *Junonia* species elsewhere is unknown.

A second mechanism for assortative mating is for different forms to become reproductively active at different times, reproducing in different years [124], at different times of the year [125], or at different times of the day [107, 126]. This reduces the likelihood of interspecific mating and permits the continued coexistence of allochronic species. There are no known diurnal differences in habitat usage among forms of *Junonia*, but there are seasonal differences that may contribute to an allochronic mechanism for persistence. In French Guiana, the foliage of *Junonia* larval host plants in the Lamiales is persistent while the foliage of larval hosts in the Scrophulariales deteriorates quickly during the dry season. The flight times of adults of *J. divaricata*, *J. evarete*, and *J. wahlbergi* coincide temporally with each other and with the presence of larval hosts in the Scrophulariales while the flight times of *J. genoveva*, *J. litoralis*, *J. neildi*, and *J. zonalis* are less seasonally restricted [23]. This difference in phenology may contribute to the continued distinctiveness of the two major *Junonia* lineages (clusters of RAF populations) in French Guiana and the French Antilles (Figure 5) but do not present an obvious mechanism for maintaining distinctive forms or species within a cluster.

A further possible mechanism may be differences in the amount or chemical composition of the pheromone or combination of pheromones used in the mating systems of different strains or species, which may allow individuals to establish a preference for other members of their own species [127]. Pheromones may differ because of intrinsic genetic differences between strains [128] or because of differences in the availability of pheromone precursors in the host plants used

by different strains [129]. Unfortunately, nothing is currently known about *Junonia* pheromone use or composition. Other characteristics of mating systems may also contribute to the assortative mating between strains or species including vocalizations, displays of colour, and physical interactions between sexes [130, 131]. Of particular interest in this regard are several characteristics of the courtship flights that are known to differ among the North American and Caribbean forms of *Junonia* [26, 108]. Variation in courtship flight patterns in other *Junonia* forms has not yet been documented. There are also differences in the colour patterns of New World *Junonia* species [22, 23, 97], but any roles that these colour pattern differences play in the mating systems are also undocumented.

Operationally, we use the isolation species concept that defines species as systems of populations such that gene exchange between these systems is limited or prevented by one or more reproductive isolating mechanisms [45, 46].

5. Conclusions

While the molecular tools employed here cannot yet distinguish between all named forms of *Junonia* we are getting much closer to having a set of reliable molecular markers for defining groups of populations within which genetic exchange is extensive and between which genetic exchange is limited, providing a means by which we can begin to distinguish between species. While *COI* barcodes are of limited utility, nuclear *wingless* sequences and RAF genotyping are effective at identifying some individual species of *Junonia* and have been very helpful in examining the relationships of *Junonia* forms from different geographic regions. Using these tools, we have determined that, in spite of phenotypic similarities, *Junonia* from the French Antilles, French Guiana, and Argentina are genetically distinct from one another and that different species likely occur in each region. *Junonia* populations also appear to cluster according to larval host plant use, supporting the hypothesis that there are two *Junonia* lineages: one which feeds primarily on plants in the order Scrophulariales and the other which uses larval host plants in the order Lamiales. The rapid growth in our knowledge of the natural and evolutionary history of New World *Junonia* in combination with the powerful experimental tools that are available for use in these organisms shows much promise in making this group an excellent model for the study of processes of speciation, host plant adaptation, and the evolution and development of colour pattern phenotypes.

Conflict of Interests

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

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Research Article

Age-Dependent Constraints of Sex Allocation in a Parasitoid Wasp

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The offspring sex ratios of parasitoid wasps often depend on the age of ovipositing females. Physiological constraints such as sperm depletion and senescence are a likely cause. Also, maternal control in response to female age may be an alternative explanation. Here valvifer or abdominal tip movements were used to assess whether age-dependent sex ratio was due to physiological constraints or maternal control with an ichneumonid wasp, *Pimpla nipponica*; the offspring sex ratio at the time of wasp emergence was compared with the sex ratio predicted from abdominal tip movements. When the female was relatively young, there was little difference between the sex ratios examined. However, as the age of the females increased, the realized offspring sex ratio at wasp emergence was more male-biased than the sex ratio predicted at the time of oviposition. Thus, there was an inconsistency between the sex ratios. Curiously, the predictions of continuous movements for male egg deposition were always perfect, regardless of maternal age; fertilization control failure was detected when the females had decided to lay female eggs. Thus, physiological constraints are a likely explanation for the inconsistency in relation to female age for *P. nipponica*.

1. Introduction

Arrhenotokous haplodiploid sex determination is common in the order Hymenoptera [1, 2]. The sex determination mechanism allows the females to intentionally control the offspring sex ratio by controlling sperm access to eggs [2, 3]. Indeed, sex ratios of many hymenopteran parasitoids are variable in response to a number of environmental factors, such as host size (= quality) and mating structure; the sex ratio variation can be understood only when we assume the maternal control of sex ratio [2–5].

A number of studies have shown that female wasps produce more male-biased sex ratio later in their life span [3, 6–8] though there was some exception [9]. The increased production of males, that is, unfertilized haploid eggs, may result from sperm depletion or reduced sperm viability. Also, control of sperm release from spermatheca may be weakened with maternal aging. In any case, physiological constraints are likely to be involved. Under some circumstances, however, maternal-age-dependent sex ratio can be an adaptive response; for example, when female wasps throughout their life have been given only large class of hosts, on which female eggs are usually laid when both small and large hosts are available, they may start to allocate male eggs in the later stage of their life [3]. If this is the case, female wasps intentionally modify the offspring sex ratio in response to their age or life expectancy.

Distinction between maternal control and physiological constraint is crucial to interpret the observed patterns of maternal-age-related sex ratio. Very few studies have provided the empirical evidence whether age-dependent sex ratio is due to maternal control or physiological constraint, however. A difficulty lies in distinguishing whether sex ratio patterns observed are caused by females' decision or by a physiological constraint such as sperm depletion. The majority of previous studies have examined the offspring sex ratio at the time of wasp emergence from the host, that is, realized sex ratio [3–5]. In some studies, sex ratios at the time of egg stage (primary sex ratio) were examined with chromosome observations [10–12] or DNA markers [13, 14] to compare with sex ratios at adult emergence (secondary sex ratio); the comparison can clarify the presence or absence of differential mortality between male and female offspring. However, such comparison does not provide any insight into

the inconsistency between maternal decision and realized sex ratio.

Although it is usually difficult to assess the maternal decision to lay male or female eggs, predicting the decision is possible at least for several species of parasitoid wasps by observing female behavior during the oviposition. The most powerful method is the direct observation of abdominal tip movements of the ovipositing female, which can reflect fertilization action during the course of egg transfer from the common oviduct to the ovipositor [15, 16]. The movements are continuous when she oviposits a male egg while they are interrupted once, an action during which sperm is probably released from spamatheca for fertilization, when a female egg is being laid. Thus, the abdominal tip movements during oviposition reflect the "decision of females."

The ichneumonid wasp *Pimpla nipponica* is a solitary idiobiont endoparasitoid attacking a variety of lepidopteran pupae [17–19] and is one of the species that show different abdominal tip movements, which enable predicting the sex of eggs that the female decides to lay [16]. Female *P. nipponica* respond to hosts of different size by changing sex allocation and allocate a higher proportion of female eggs in larger hosts [10, 12].

Here, the abdominal tip movements of *P. nipponica* were used to examine whether the offspring sex ratio at the time of wasp emergence was consistent with the sex ratio determined by maternal decisions. Particularly, the present study focused on a change of the sex ratio inconsistency in relation to the age of the ovipositing female. Based on the results, the evidence is given for the presence of sex ratio constrained by physiological causes.

2. Materials and Methods

2.1. Parasitoid and Host. A laboratory colony of *P. nipponica* was established using adult parasitoids collected from Nagoya City, Japan. Female *P. nipponica* were placed individually in transparent plastic cups (10 cm in diameter, 4.5 cm in height), together with tissue paper saturated with diluted honey. The tissue paper was replaced twice a week thereafter. The cups were kept at 20 ± 1°C with a 16L:8D photoperiod. The colony was maintained on pupae of a laboratory host, *Galleria mellonella*. Host cocoons containing fresh pupae were presented to the females in the plastic cups for parasitization. Parasitized hosts were removed from the cups and were held at 25 ± 1°C with a 16L:8D photoperiod, until parasitoid emergence. Newly emerged parasitoids were paired and placed in the plastic cups and were maintained as mentioned above.

2.2. Sex Allocation Experiment. The sex of the offspring that *P. nipponica* decided to produce upon oviposition was predicted on the basis of the abdominal tip movements during oviposition (i.e., primary sex ratio) whereas sex ratios that were realized were examined at the time of offspring wasp emergence (i.e., secondary sex ratio). The movements may be continuous, or be interrupted once, during egg deposition, and the former movements indicate deposition of unfertilized eggs and the latter fertilized female eggs [16].

Newly emerged females were paired with a male in transparent plastic cups and observed to confirm successful mating. Care was taken to avoid sib-mating by using non-sib males. After mating, males were removed from the cups. Female *P. nipponica* for testing were reared at 20 ± 0.5°C, 16L:8D, during the experimental period. Twenty-two females in all were used for the experiment. Two host cocoons were given to the females in the cups for 2 hours twice a week after wasp emergence until death as a conditioning treatment. This treatment was needed to keep the females reproductive; an interruption of host provision for a relatively long period of time, for example, 1 or 2 weeks, can cause them having no eggs ready for oviposition (Ueno, personal observations).

Data were collected by arbitrarily giving 1-2 hosts to test females of 3–49 days old (the longevity of female *P. nipponica* is normally 35–45 days in the laboratory condition). Host cocoons containing a fresh pupa were offered singly to test females, and oviposition in the hosts was directly observed under a stereoscopic binocular microscope, recording abdominal tip movement patterns. Each female was allowed to attack a host only once to avoid superparasitism. Then, parasitized hosts were collected and kept singly at 25 ± 1°C, 16L:8D, until wasp emergence. When the offspring wasps emerged, the sex was recorded. Hosts that did not produce any wasp after 28–30 days since parasitization were discarded because the offspring wasps normally emerged within 3 weeks since parasitization [19].

Data were analyzed with the aid of JMP version 9.0 (SAS Institute, 2010). Fisher's exact probability test was used to evaluate the inconsistency of sex ratios. A logistic regression analysis was applied to examine the presence of age-related inconsistencies between predicted and realized sex ratios. This analysis was made with the data set for interrupted movements, in which production of male offspring was assumed to imply fertilization failure. Production of male or female offspring was a categorical dependent variable (binary variables) whereas maternal age was used as a continuous independent variable.

3. Results

In all, 196 hosts presented to test femaleswere parasitized. In 7 cases, the abdominal tip movements during oviposition were not recorded because the females did not oviposit an egg soon after the insertion of the ovipositor and became motionless with the ovipositor inserted in host. In 9 cases, judgment was difficult to classify the abdominal movements into continuous or interrupted because of the angle of vision for the observer. The above cases were excluded from the following analyses.

In all, continuous and interrupted movements were recorded for 85 and 95 parasitized hosts, respectively. Of these, 66 and 80 hosts, respectively, produced the offspring wasps. The results confirmed that the continuous and interrupted abdominal movements were mostly linked to the production of male and female offspring, respectively

Table 1: Accuracy of abdominal tip movements for predicting the sex of offspring produced.

Movements	N	% wasp emergence	Number of females produced	Number of males emerged	Sex ratio (% females)
Continuous	85	77.6	0	66	0
Interrupted	95	84.2	67	13	83.8

(Table 1). Maternal control was perfect when female *P. nipponica* decided to lay male eggs (Table 1). By contrast, in 13 out of 80 cases (16.3%), males were produced despite the interrupted movement. The result suggested that the failure of fertilization occurred even when the females tried to release sperm and fertilize eggs to produce female offspring. When all data were pooled regardless of female age, the percentage of failure of fertilization control differed significantly between the continuous and interrupted movements (Fisher's exact probability test; df = 1, $P = 0.0003$). In addition, the failure of fertilization control was more likely to take place in older females (Figure 1). A logistic regression analysis revealed that the likelihood of fertilization control failure significantly increased with female age when the females tried to lay female eggs (df = 1, $x^2 = 4.72$, $P = 0.029$). The results demonstrated the presence of physiological constraints associated with female age.

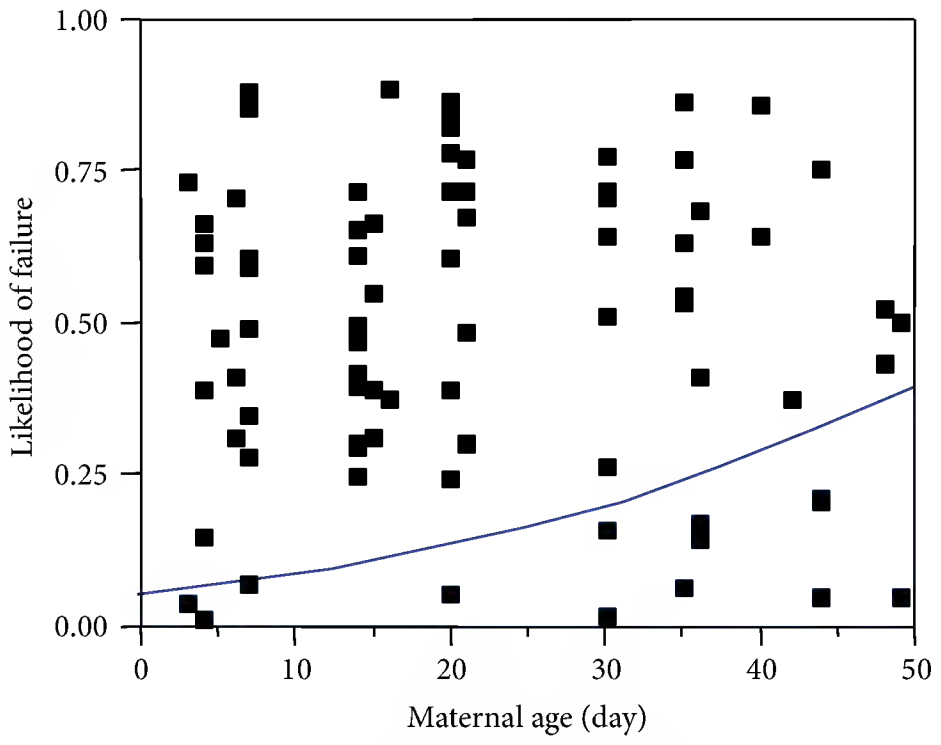

Figure 1: A logistic regression line obtained for the relationship between maternal age and likelihood of fertilization failure. The points are placed midway to assure their visibility, and the horizontal placement of each point (= one oviposition event) is made with respect to the horizontal axis (= female wasp age). The points above or below the curve show data set in which test females produced female or male offspring (see JMP User's Guide).

4. Discussion

The present study has confirmed that abdominal tip movements are good predictors of the sex of depositing eggs. The movements, however, are not always perfect indices; male eggs were occasionally produced even when female *P. nipponica* showed interrupted movements. This inconsistency between the female's decisions and the realized sex of the offspring can be caused by physiological constraints. Constrained sex ratio in parasitoids should occur when females are unmated, sperm-depleted, senescent, or multiply mated [20–23] or male counterparts are of low quality [24, 25]. Females may be sperm-depleted in the late stage of their life when they have laid many fertilized eggs. Sperm depletion can also take place when females mate with low-quality males (e.g., small males) or multiply mated males [23, 25].

In the present study, evidence has shown that constrained sex ratio occurs for senescent *P. nipponica*; with increasing the age, the females were more likely to lay unfertilized male eggs despite their decisions to fertilize depositing eggs. This appears to be due to sperm depletion or physiological constraints associated with senescence. It also implies that an ovipositing female did not have a mechanism for assessing her physiological state.

In several parasitoids, the behavior of unmated females differs from that of mated females, suggesting that females can recognize their mating status [26, 27]. In *P. nipponica,* however, unmated females behave as mated females; they show interrupted movements though they have no sperm in the spermatheca [16]. It is, hence, unlikely that female *P. nipponica* can recognize the number of sperms in the spermatheca; the senescent females would not modify their sex allocation in response to sperm depletion.

Curiously, senescence did not influence the consistency between the continuous movements and the deposition of unfertilized male eggs. This result indicates that sperm is never wrongly released regardless of female age. I suggest that the exit of sperm from spermatheca is normally closed, and senescence affects only the control to open the exit for sperm release to fertilize the eggs.

Parasitoid wasps have been shown to be excellent organisms for testing sex ratio theory [1, 4, 12, 28]. However, sex ratio theory generally assumes perfect maternal control over the offspring sex allocation. In fact, constrained sex ratio does occur in several parasitoid wasps, mostly due to unmatedness, and the presence of constrained unmated females should lead to the production of a more female-biased sex ratio by nonconstrained mated females than predicted [20–22]. This means that constrained females influence the sex allocation decision of other nonconstrained females.

Likewise, senescent *P. nipponica* females can be constrained from perfectly controlling the offspring sex ratio, and the consequence is the production of large males from large hosts, which should normally produce female offspring. The presence of constrained females may affect the overall sex

ratio produced by nonconstrained females, and the latter may produce a more female-biased sex allocation to a given size of hosts than predicted. However, in the field, females should have a shorter life span than physiologically attainable span, and females that are constrained due to senescence should be rather rare. Given this, the impact of constrained females may not be large. In any case, the effect of constrained females on the sex ratio produced by nonconstrained females would be a future subject.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research Article

Monitoring Spruce Budworm with Light Traps: The Effect of Trap Position

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Daily records of adult spruce budworms, *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae), captured at light traps at multiple locations in New Brunswick in the 1970s, are analyzed in relation to the physical position of light traps (tree canopies or forest clearings). Captures at light traps deployed in tree canopies were 4–400 times greater than those in forest clearings, especially for males. The phenology of captures (median date or duration of flight period) did not differ in relation to trap location. Captures of both males and females in tree canopies were highly correlated with egg densities, whereas no significant relationship was observed for either sex in forest clearings. Monitoring programs for spruce budworm adults using light traps should be standardized by deploying traps in tree canopies.

1. Introduction

The spruce budworm (SBW), *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae), is the most severe defoliator of conifers in the Nearctic boreal forest [1, 2]. Population dynamics of SBW are strongly influenced by long-range migration of adults that ascend to the atmospheric boundary layer to be carried downwind [3]. Understanding dispersal-migration in SBW is important to forecast the spatiotemporal occurrence of outbreaks and implement sound pest management strategies [4, 5].

In the 1980s, monitoring procedures to assess the abundance of adult SBW shifted from light traps [6, 7] to traps baited with female sex pheromone [8]. Captures of SBW at light traps are sensitive to yearly variations in population density [9, 10] and useful for detecting mass-immigration events [11, 12]. The shortcoming of light traps is that multiple species are attracted, requiring a time-consuming process to sort SBW from other moths. This explains the replacement of light traps by pheromone traps, which are selective in capturing only SBW. During the transition between monitoring procedures, no comparison was made between captures at light traps and pheromone traps; thus, it is not possible to assess the relative performance of these two approaches.

One important difference between light traps and pheromone traps is that the former capture both males and females, whereas the latter capture only males. Strict reliance on pheromone trapping of males may yield inconsistent predictions relative to larval abundance or defoliation intensity because damage is ultimately caused by egg-carrying females and not mate-seeking males [13]. An implicit yet rarely tested assumption underlying monitoring populations with pheromone traps is that the spatiotemporal distributions of mate-seeking males mirror those of ovipositing females; this assumption is probably *not* valid in SBW due to sex differences in dispersal behavior [3]. These considerations may explain apparent limitations of pheromone trapping as a monitoring tool, for example, poor estimates of larval densities or future defoliation (low r^2 values of regression models) [14, 15], and yearly variation in the relationship between trap captures and larval density [16].

With the looming SBW outbreak soon expected to rage across Atlantic Canada [17], monitoring of adults in future research programs will include an extensive network of both light traps and pheromone-baited traps [http://www.healthyforestpartnership.ca/research-in-action/research-program-overview; accessed 18 August 2014]. One important issue

to settle before implementing broad-scale light trapping of SBW is standardization of the physical position of light traps in forest habitats. In the past, light traps have been indiscriminately deployed in tree canopies or forest clearings without recording the position of traps [6, 7].

Empirical data sources collected in New Brunswick in the 1970s and replicated across multiple locations are summarized here, with the objective of comparing the abundance, sex ratio, and phenology of adult SBW captured at light traps deployed in forest clearings and tree canopies.

2. Materials and Methods

The experiment was conducted at four sites in New Brunswick, Canada: Acadia Forest Experiment Station near Fredericton in 1972 and 1976; Chipman in 1973, 1974, and 1975; Juniper in 1975; and Saint-Quentin in 1977 (see [18] for geographic location of sites). The sites consisted of forest stands containing hosts of SBW (balsam fir, *Abies balsamea* (L.) Mill., and spruces, *Picea* spp.) as either dominant or codominant tree species.

The light source used to attract moths consisted of a Coleman lantern powered by naphtha gas and operated from sunset until dawn, placed at the center of two crossing vanes that deflected the moths down toward a funnel into the trap; a strip of insecticide (Vapona) was placed at the bottom of the trap to kill captured insects [6]. At each site, one light trap was suspended in the upper canopy of host trees (>6 m high) and the other was deployed 2 m above ground from a tripod in a forest clearing; the distance between edges of forest clearings and traps in tree canopies was >50 m at all sites. The traps were inspected daily, and the captured SBW moths were collected to determine abundance of males and females. The same design was used in Saint-Quentin, except that light traps were deployed at nine plots spaced 3-4 km apart along a logging road [19].

Egg density was estimated on the most prevalent host trees of SBW at different sites by pruning one branch (usually <1 m long) in the midcanopy of three to five codominant trees and estimating the number of egg masses visually; the surface area of branches was estimated (length × width of midpoint branch section) and the density of eggs was expressed in terms of egg masses per 10 m^2 branch area [2].

Statistical analyses were conducted with the SAS package (SAS Institute, Cary, NC). Three parameters related to phenology were calculated for each site and trap location: (1) the median date of captures; (2) the duration of flight (interval in days between the 5th and 95th cumulative captures dates); and (3) the proportion of individuals captured at light traps during the modal date of abundance. The cumulative abundance, sex ratio, and phenology of SBW were compared in relation to trap position at different sites using paired t-tests; at Saint-Quentin, pooled values across plots were used in analysis.

The relationship between cumulative captures of males and females and the density of eggs per 10 m^2 of foliage at different sites was evaluated in relation to sex and trap position using regressions. In addition to the seven aforementioned sites, the regression models also included two sites sampled in 1978 (Saint-Quentin and Amherst, Nova Scotia) for which the cumulative (but not daily) abundance of SBW males and females at light traps was available; therefore, the regression models each included nine data points.

Data were subjected to logarithmic (abundance data) and arcsine (proportion data) transformation to reduce heterogeneity of variance.

3. Results

Daily numbers of males and females at TC and FC are plotted for different locations in Figure 1. Captures of adults at TC were 4–400 greater than those at FC (Table 1). The sex ratio of moths at TC was strongly biased toward males, whereas the sex ratio at FC was approximately even. Neither the median date of flight nor the duration of the flight period varied significantly with trap position (Table 1). The proportion of adults captured during the modal date of flight was two times higher at FC than TC (Table 1).

The density of eggs at different sites varied from 36 to 992 egg masses per 10 m^2 of foliage. Numbers of males and females captured at TC were positively correlated with the density of eggs, whereas no significant relationship was observed at FC (Figure 2).

4. Discussion

With the imminent SBW outbreak in Atlantic Canada, it is important to consolidate and disseminate the information gathered by research scientists during previous outbreaks, in particular as-yet unpublished data. Results obtained through this study are published for the first time and provide a detailed record of daily captures of male and female SBW at light traps deployed in forest clearings or tree canopies at multiple locations. The findings have important implications for the design of monitoring procedures and analysis of time-series data and also provide insight into the dispersal-migration behavior of SBW.

The physical position of light traps must be standardized in monitoring procedures for SBW. Deployment of traps in forest clearings should be avoided due to the low numeric abundance of SBW and the insignificant relationship between moth abundance and egg densities (Table 1, Figure 2). Captures of SBW at light traps in tree canopies, in contrast, provide accurate estimates of egg densities (Figure 2). The relationships were similar for males and females; thus, the time-consuming process of sexing SBW could be omitted in operational programs that are purely management oriented. From a research perspective, recording the sex ratio of moth captures is useful to unravel the role of dispersal-migration on SBW population dynamics. Ideally, both light traps and pheromone-baited traps should be emptied daily or at short intervals to reduce the risk of trap saturation and gain information related to flight phenology and immigration events (nightly captures are characterized by high numerical abundance, predominantly females) [3, 9, 11, 12].

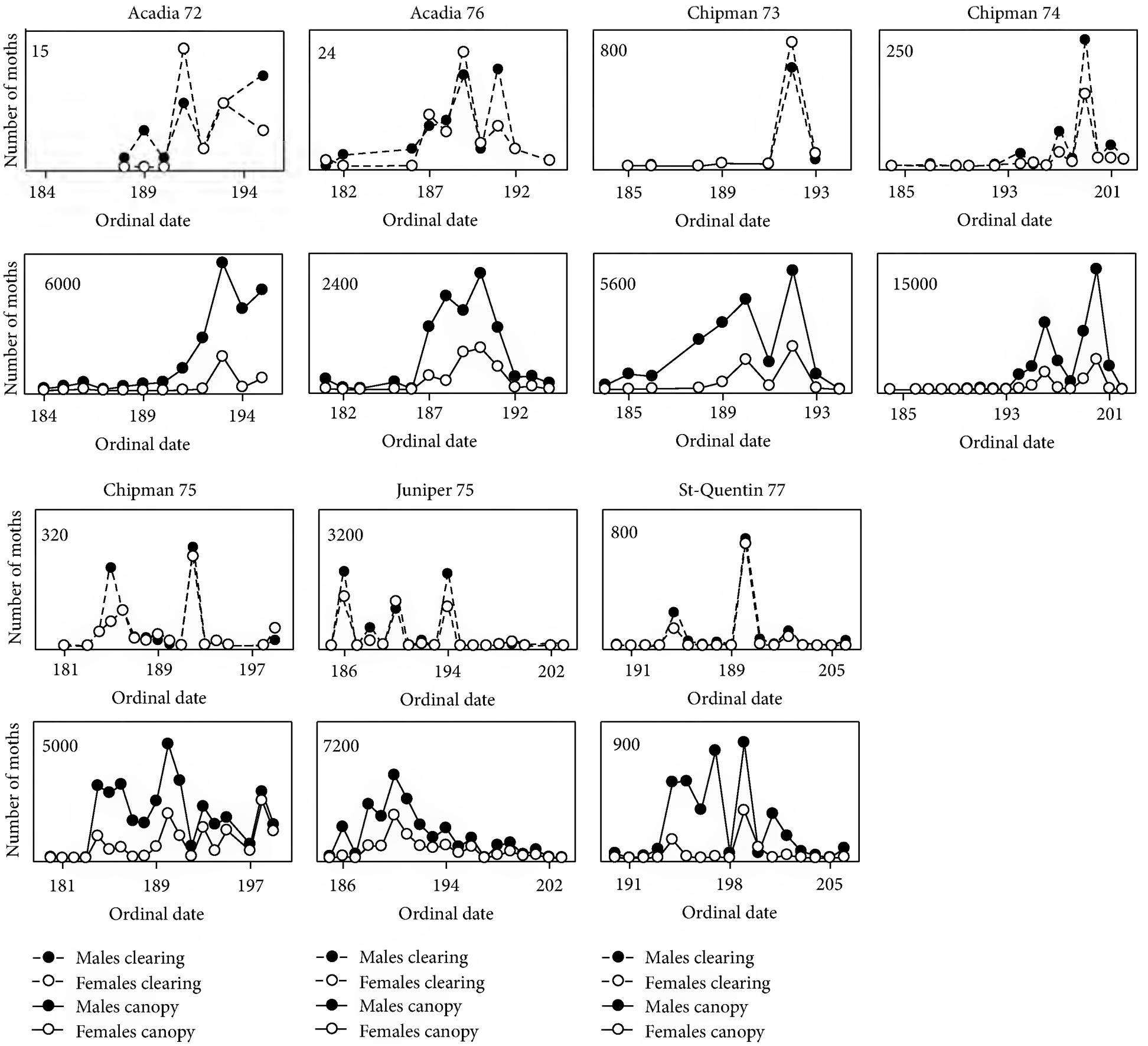

FIGURE 1: Daily abundance of adult spruce budworms captured at light traps deployed in tree canopies and forest clearings at different sites in New Brunswick, Canada. The numerical values within individual graphs represent the scale of the y-axis for different locations and trap position.

TABLE 1: Descriptive parameters related to captures of adult spruce budworms at light traps deployed in tree canopies and forest clearings at seven locations—years (mean ± SE). For each parameter, values with different superscripts are significantly different (paired t-tests, $P < 0.05$). Data were subjected to logarithmic (number of moths) and arcsine (proportional data) transformations to reduce heterogeneity of variance.

Independent variable	Position of traps		t-test	
	Tree canopy	Forest clearing	t	P
Abundance of moths (1000)	24.5 ± 5.6^{a}	1.9 ± 1.1^{b}	5.02	0.0024
Sex ratio (P_{females})	0.193 ± 0.02^{a}	0.444 ± 0.024^{b}	7.51	0.0003
Median date of flight	192.4 ± 1.5^{a}	192.7 ± 1.6^{a}	0.26	0.8041
Duration of flight (day)	8.7 ± 1.3^{a}	7.2 ± 1.1^{a}	1.20	0.2741
Proportion of adults (modal date)	0.251 ± 0.026^{a}	0.514 ± 0.077^{b}	3.71	0.0100

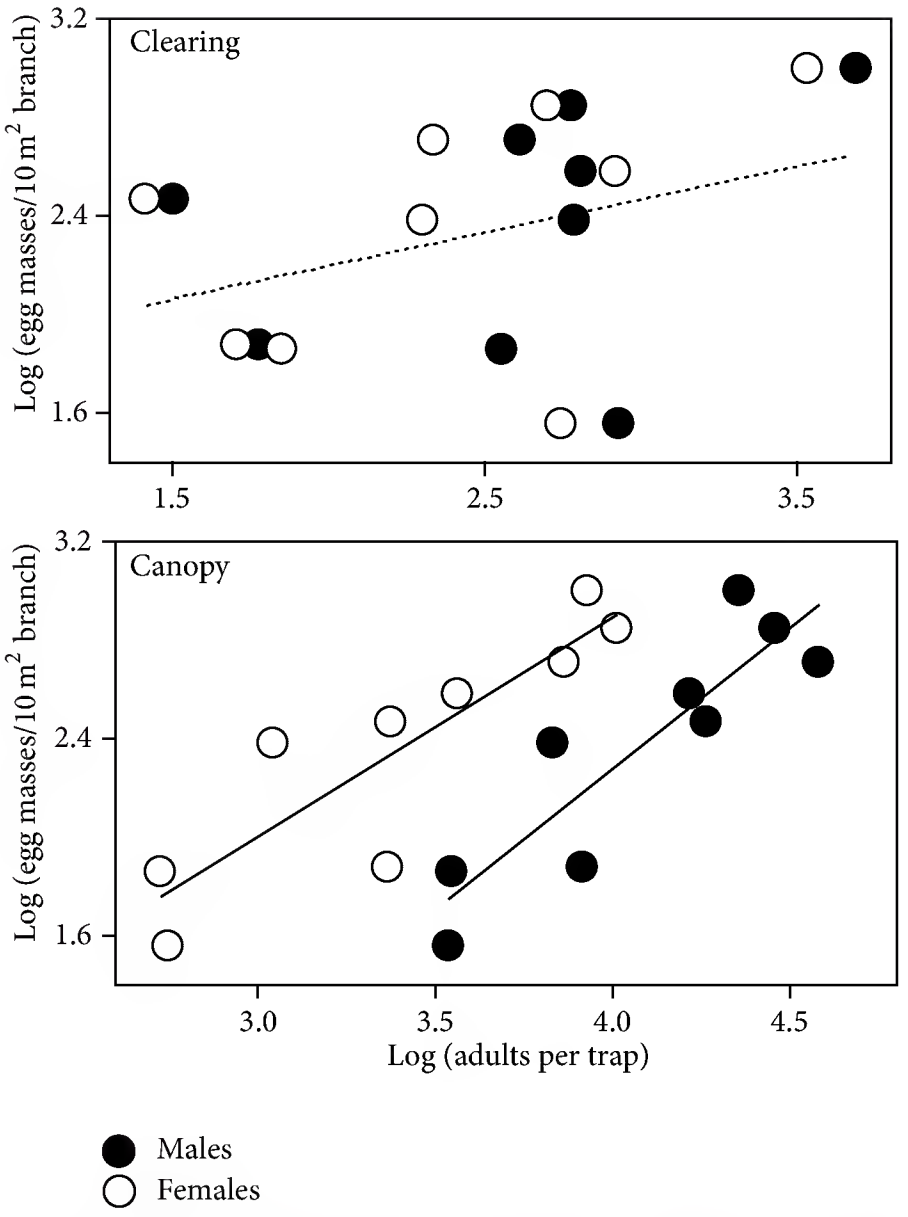

FIGURE 2: Relationships between numbers of adult spruce budworms captured at light traps deployed in tree canopies and forest clearings (x-axis) and the density of eggs (y-axis) at nine locations in New Brunswick, Canada, in the 1970s. Regressions based on captures in tree canopies were statistically significant for both males ($y = 1.14x - 2.30$, $r^2 = 0.796$, and $P = 0.0012$) and females ($y = 0.89x - 0.67$, $r^2 = 0.776$, and$P = 0.0017$) (solid lines). Regressions based on captures in forest clearings were not significant for either sex (males: $y = 0.27x + 1.67$, $r^2 = 0.119$, and $P = 0.3626$; females: $y = 0.34x + 1.56$, $r^2 = 0.205$, and $P = 0.221$) (dotted lines). Data were subjected to logarithmic transformations to reduce heterogeneity of variance.

Daily records of moth captures at light traps were kept for multiple locations in Atlantic Canada and Maine in the 1970s [6, 7], which might in theory provide extensive time-series analysis of moth abundance. These data should *not* be analyzed in this context, however, because the position of traps is unspecified for most sites; thus, sampling artefacts (captures are 4–400 times greater in tree canopies than forest clearings) would systematically bias the outcome of analyses. The data are apparently suitable for evaluating broad-scale trends related to the phenology of flight in SBW, because neither the median date nor the duration of flight periods is influenced by the position of traps (Table 1). Existing phenology models of SBW adult flight need to be validated and calibrated with field data because they do *not* appear to accurately reflect the timing of SBW flight (Figure 6 in [20]).

It has been hypothesized that light traps deployed in forest clearings capture predominantly dispersive SBW, whereas traps deployed in tree canopies capture local moths [3, 9, 10, 12]. Although this hypothesis cannot be unambiguously tested (because no genotypic-phenotypic traits are available to distinguish migrant from resident adults), its framework can be used to make *a priori* predictions related to patterns of captures of SBW in traps deployed in tree canopies and forest clearings, specifically: (1) greater captures in tree canopies than forest clearings (due to the greater number of resident moths than dispersers in forest stands with high densities of SBW); (2) greater proportion of females in forest clearings than tree canopies (because females are more likely to disperse than males); (3) shorter durations of moth activity in forest clearings than tree canopies (due to the transient nature of dispersal events relative to patterns of emergence-activity of resident moths); and (4) greater proportion of moths captured during the modal (peak) date of captures for forest clearings than tree canopies (transience of dispersal events). The data provided strong statistical support for three of the four predictions (Table 1), suggesting that light traps in forest clearings are indeed more likely to capture dispersive SBW than light traps in tree canopies.

Conflict of Interests

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

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Research Article

Chemical Composition and Acaricidal Effects of Essential Oils of *Foeniculum vulgare* Mill. (Apiales: Apiaceae) and *Lavandula angustifolia* Miller (Lamiales: Lamiaceae) against *Tetranychus urticae* Koch (Acari: Tetranychidae)

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Utilization of synthetic acaricides causes negative side-effects on nontarget organisms and environment and most of the mite species such as two spotted spider mite, *Tetranychus urticae* Koch, are becoming resistant to these chemicals. In the present study, essential oils of fennel, *Foeniculum vulgare* Mill., and lavender, *Lavandula angustifolia* Miller, were hydrodistilled using Clevenger apparatus and chemical composition of these oils was analyzed by GC-MS. Anethole (46.73%), limonene (13.65%), and α-fenchone (8.27%) in the fennel essential oil and linalool (28.63%), 1,8-cineole (18.65%), and 1-borneol (15.94%) in the lavender essential oil were found as main components. Contact and fumigant toxicity of essential oils was assessed against adult females of *T. urticae* after 24 h exposure time. The essential oils revealed strong toxicity in both contact and fumigant bioassays and the activity dependeds on essential oil concentrations. Lethal concentration 50% for the population of mite (LC_{50}) was found as 0.557% (0.445–0.716) and 0.792% (0.598–1.091) in the contact toxicity and 1.876 μL/L air (1.786–1.982) and 1.971 μL/L air (1.628–2.478) in the fumigant toxicity for fennel and lavender oils, respectively. Results indicated that *F. vulgare* and *L. angustifolia* essential oils might be useful for managing of two spotted spider mite, *T. urticae*.

1. Introduction

Spider mites belong to the family Tetranychidae and are named because many members of this family produce silk webbing on the host plants. Some 1,200 species of spider mites belonging to over 70 genera are known in the world especially in the Southern Hemisphere [1]. Two-spotted spider mite, *Tetranychus urticae* Koch, is widely distributed globally and a common pest of many plant species in greenhouses, orchards and field crops. To date, 3877 host species have been reported around the world in both outdoor crops and greenhouses [2]. *T. urticae* feeding causes graying or yellowing of the leaves and necrotic spots occur in advanced stages of leaf damage. Mite damage to the open flower causes a browning and withering of the petals that resembles spray burn. In addition, small chlorate spots can be formed at feeding sites as the mesophyll tissue collapses due to the destruction of 18–22 cells per minute [2]. The importance of this mite pest is not only due to direct damage to plants but also due to indirect damage to plants which decreases photosynthesis and transpiration [3]. Because of their high reproductive rates, its management can be difficult. When mites begin to feed on a plant, they produce webbing that can protect both motile and egg stages from the acaricide [3].

Synthetic acaricides have been used as the main strategy for *Tetranychus* species resulting in an increased cost for production and environmental impacts as well as resistance development to even the newly synthesized molecules such abamectin [2, 4]. In addition, control methods based on the use of synthetic acaricides sometimes fail to keep the number

of spider mites below economic threshold levels [5]. It is therefore necessary to find alternatives that can minimize negative effects of synthetic acaricides.

Essential oils obtained by hydrodistillation, steam distillation, dry distillation, or mechanical cold pressing of aromatic plants [6] have long been used as fragrances and flavorings in the perfume and food industries, respectively, and recently for aromatherapy and medicines [2]. The essential oils can play major roles in pollination by attracting insects and in water loss's prevention due to excessive evaporation. Repellence is another property of essential oils, as some contain numerous secondary metabolites that can deter attacks from pests [7]. Most essential oil constituents degrade quickly in the environment or are rapidly lost from plant foliage through volatilization, which minimizes residual contact. They have short residual activities due to temperature and UV light degradation and, with a few exceptions, their mammalian toxicity is low [8, 9]. Therefore, essential oils can be applied to both field and greenhouse crops in the same manner as current synthetic acaricides [2, 9].

The fennel, *Foeniculum vulgare* Mill. [Apiales: Apiaceae (Umbelliferae)], is indigenous to the Mediterranean and is largely used to impart flavor to a number of foods, such as soups, sauces, pickles, breads, and cakes. It is an annual, biennial, or perennial herbaceous plant, depending on the variety, which grows in good soils from sunny mild climatic regions and is a well-known aromatic plant species. Traditionally in Europe and Mediterranean areas, fennel is used as antispasmodic, diuretic, anti-inflammatory, analgesic, secretolytic, galactagogue, eye lotion, and antioxidant remedy [10, 11]. The lavender, *Lavandula angustifolia* Miller [Lamiales: Lamiaceae (Labiatae)], is an evergreen bushy shrub with straight, woody branches; the lower parts are leafless, putting out numerous herbaceous stems to a height of about 1 m [12]. It is native to southern Europe and the Mediterranean area and is commercially cultivated in France, Spain, Portugal, Hungary, UK, Bulgaria, Australia, China, and USA [13].

This paper describes a laboratory study examining the contact and fumigant toxicity of essential oils of *F. vulgare* and *L. angustifolia* grown in Iran against of *T. urticae* followed by evaluation of their chemical constituents by Gas chromatography-Mass spectrometry (GC-MS).

2. Materials and Methods

2.1. Rearing of Two-Spotted Spider Mite. The two-spotted spider mites were collected from infested leaves of some wildly grown weeds in the yard of University of Mohaghegh Ardabili which did not have any exposure to acaricides. The *Tetranychus urticae* Koch species after separation, and slide preparation was identified according to introduced keys by Zhang [1]. Spider mites were reared on navy bean (*Vigna unguiculata* Walp. [Fabales: Fabaceae]) plants for one year. The infested plants were held in cages (120 × 300 × 100 cm) covered with mesh cloth. To synchronize the adult stage of *T. urticae* for adulticidal bioassays, 50 adult female mites were transferred to the leaves of trifoliate bean plants (held individually in cylindrical glass containers with appropriate aeration) with a hair brush and allowed to lay eggs for 24 h, after which the adults were removed. The infested leaves were held at the above-mentioned conditions to allow the eggs to hatch and the larvae to develop into synchronized adults. All experiments were carried out at 25 ± 2°C, 60 ± 5% relative humidity (RH) and a photoperiod of 16 : 8 (light : dark) in a growth chamber.

2.2. Plant Materials and Essential Oil Extraction. Aerial parts 5 cm from the top of *L. angustifolia* at flowering stage and seeds of *F. vulgare* were collected from Ardabil, Ardabil province, Iran from June to August 2013. The specimens were air dried in the shade at room temperature and chopped into small pieces with electric grinder. The essential oils were extracted using a Clevenger-type water steam distillation apparatus within 3 h. To carry out the extraction, 100 g of powdered plant material was used along with 1200 mL distillated water. Anhydrous sodium sulphate was used to remove excess water after extraction. The essential oils were transferred to dark brown glass vials covered with aluminum foil and stored in refrigerator at 4°C until used in the experiments.

2.3. Analysis of Essential Oil. One μL of prepared essential oil was injected to GC-MS (HP Agilent 6800N/(61530N) with CPSil5CB column (Chrompack, 100% dimethyl polysiloxane 60 m, 0.25 mm (ID) film thickness 0.25 l). The analysis was performed under temperature programming from 100°C (3 min) to 250°C (5 min) with the rate of 3°C/min. Injector temperature was 230°C. Identification of spectra was carried out by study of their fragmentation and also by comparison with standard spectra present in the library of the instrument. Area normalization was used for determination of composition percentage.

2.4. Contact Toxicity. Contact toxicity was conducted in Petri dishes (6 cm diameter). Concentrations ranged from 0.12% to 2.8% for *L. angustifolia* and 0.11% to 1.7% for *F. vulgare*, using a spreader sticker adjuvant (20 μL Tween, 0.02%) diluted in distilled water. Leaf discs (3 cm diameter) were cut from leaves of greenhouse-grown *V. unguiculata* and immersed in solutions of the each essential oil for 20 seconds. After drying at room temperature for 45 min, each disc was individually placed at the bottom of a Petri dish atop a 6 cm diameter disc of filter paper wetted with distilled water. The wet cotton pads were then placed on excised leaves and ten adult females were transferred thereafter. The lids of Petri dishes were pierced (1 cm in diameter) and their openings were covered with mesh cloth in order to evade of fumigant toxicity. Control mites were held on leaf discs immersed in dilutions without essential oils. Mortality was noted after 24 h and there were tree replicates for each treatment. Mites were considered dead if appendages did not move when prodded with a fine paintbrush.

2.5. Fumigant Toxicity. For evaluation of fumigant toxicity, 750 mL plastic containers with tight lids were used as the test chambers. Each treatment consisted of five concentrations of the essential oil and a control. Based on preliminary experiments, ranges of concentrations tested against the adult

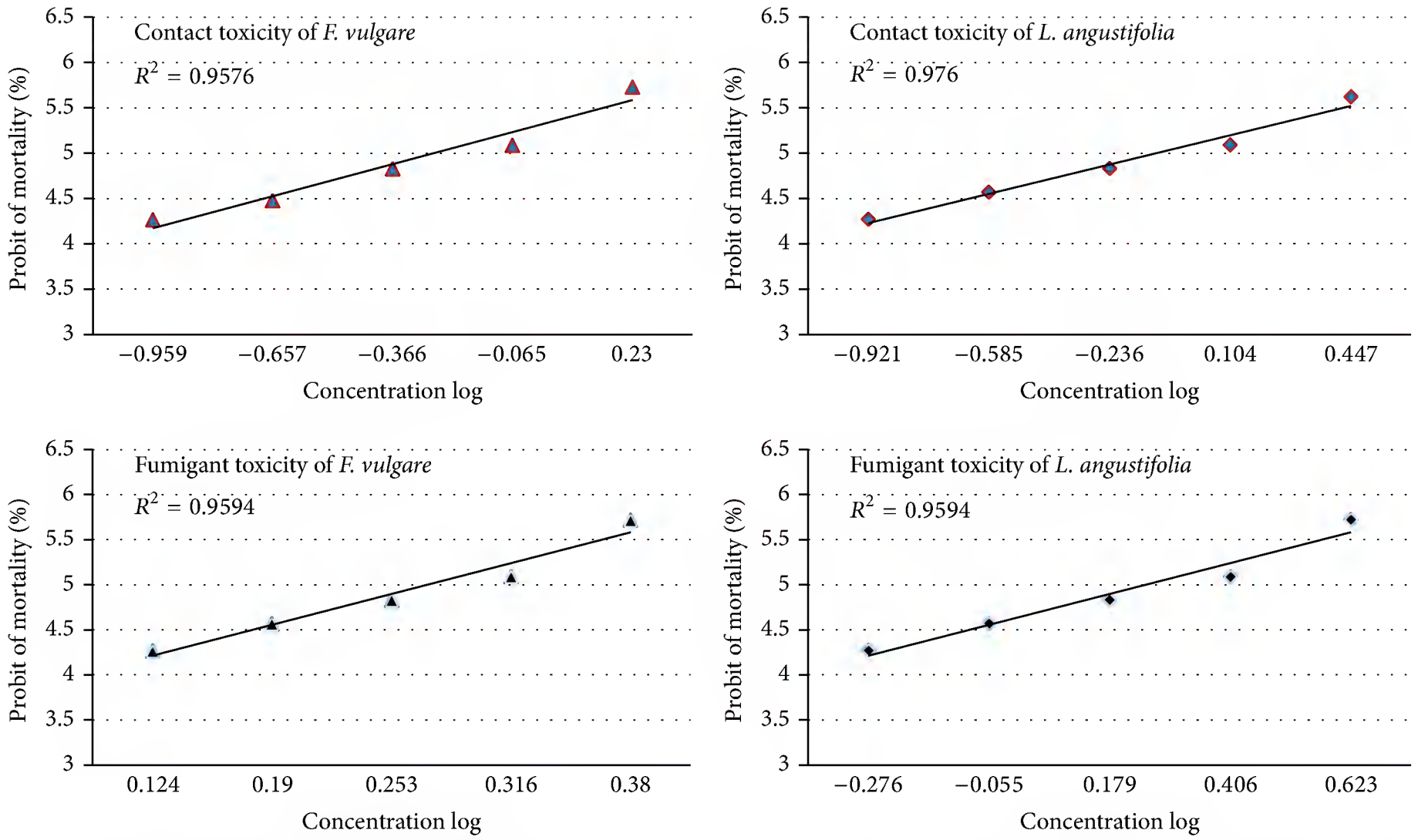

FIGURE 1: Concentration-mortality response lines for adult females of *Tetranychus urticae* exposed to different concentrations of *Foeniculum vulgare* and *Lavandula angustifolia* essential oils.

females were 0.53 to 4.2 and 1.33 to 2.4 μL/L air for *L. angustifolia* and *F. vulgare*, respectively. For each concentration, three replicates were used. Each replicate consisted of sixty 24 h old adult females on each leaf plant disc. The discs (3 cm in diameter) punched from leaves of bean plants were placed inside a 6 cm diameter plastic Petri dishes without lids lined with water-soaked cotton. The Petri dishes were then put into plastic containers used as fumigation chambers. To achieve the desired concentration of the oil in the fumigation chambers, using a micropipette, the appropriate volume of the oil was applied on a 2 × 2 cm strip of Whatman no. 1 filter paper adhered to the inner surface of the fumigation chamber. The exposure period for assessing the adulticidal effect of the essential oils was 24 h. To determine mortality, the mites were touched with the tip of a fine hair brush. If the mite did not move, it was considered dead. The controls consisted of the same number of mites as the treatments; and were kept under the same conditions on leaf disks left untreated.

2.6. Analysis of Data. Experiments were arranged in a completely randomized design and data were analyzed by ANOVA. The mortality data were subjected to probit analysis using SPSS software to estimate LC_{50} values of the essential oils against *T. urticae*.

3. Results

Using hydrodistillation process, fennel seed yielded 2.15% essential oil while lavender leaf yielded 2.01%. Results of analysis of the essential oils are presented in Table 1. Twenty six compounds were identified in the essential oil of fennel, representing 99.94% of the total essential oil sample while twenty five compounds were found in the lavender essential oil, representing 99.97% of the total essential oil sample. The major components were found to be anethole (46.73%), limonene (13.65%), α-fenchone (8.27%), carvone (6.12%), and estragole (5.26%) for *F. vulgare* essential oil and Linalool (28.63%), 1,8-Cineole (18.65%), 1-Borneol (15.94%), Camphor (8.20%), and Terpineol-4 (4.27%) for *L. angustifolia* essential oil. Amounts of monoterpenes hydrocarbon in the essential oils of *F. vulgare* and *L. angustifolia* were 20.43% and 8.55%, respectively. Monoterpenoids content of *F. vulgare* and *L. angustifolia* essential oils was 20.27% and 83.36% and the sesquiterpenes were 0.44% and 2.38%, respectively.

F. vulgare and *L. angustifolia* essential oils revealed strong significant toxicity on the adult females of *T. urticae* in both contact and fumigant assays. The activity depended on essential oil concentrations in both contact and fumigant bioassays and increased susceptibility of mite was directly associated with oil concentration (Table 2 and Figure 1).

In the contact toxicity, lethal concentration 50% mite mortality (LC_{50}) was 0.557% and 0.792% with *F. vulgare* and *L. angustifolia*, respectively, with *F. vulgare* essential oil being the most toxic the adult females of *T. urticae* (Table 2). On the other hand, fumigant toxicity was 1.876 and 1.971 μL/L air for *F. vulgare* and *L. angustifolia* essential oils, respectively (Table 2).

4. Discussion

In the present study, anethole, limonene, α-fenchone, and carvone were the major compounds of essential oil of

Table 1: Chemical analysis of essential oils of *Foeniculum vulgare* and *Lavandula angustifolia* grown in Iran by GC-MS.

Compound	*F. vulgare*		*L. angustifolia*		Formula	Molecular weight (g/mol)	Classification
	RT	Percentage	RT	Percentage			
α-Pinene	3.93	3.53	4.80	1.46	$C_{10}H_{16}$	136.23	Monoterpene
Camphene	4.17	0.32	5.08	0.68	$C_{10}H_{16}$	136.23	Monoterpene
Sabinene	4.51	0.67	5.54	0.96	$C_{10}H_{16}$	136.23	Monoterpene
β-Pinene	4.61	0.20	5.64	2.16	$C_{10}H_{16}$	136.23	Monoterpene
Myrcene	4.74	1.09	5.84	1.39	$C_{10}H_{16}$	136.23	Monoterpene
α-Phellandrene	5.05	0.54	—	—	$C_{10}H_{16}$	136.23	Monoterpene
Limonene	5.61	13.65	—	—	$C_{10}H_{16}$	136.23	Monoterpene
1-Octen-3-ol	—	—	5.73	0.46	$C_8H_{16}O$	128.21	Alcohol
γ-Terpinene	6.06	0.43	—	—	$C_{10}H_{16}$	136.23	Monoterpene
δ-3-Carene	—	—	6.27	0.72	$C_{10}H_{16}$	136.23	Monoterpene
α-Fenchone	6.83	8.27	—	—	$C_{10}H_{16}O$	152.23	Monoterpenoid
1,8-Cineole	—	—	6.87	18.65	$C_{10}H_{18}O$	154.25	Monoterpenoid
trans-β-Ocimene	—	—	7.10	0.98	$C_{10}H_{16}$	136.23	Monoterpene
Terpineol-4	—	—	7.58	4.27	$C_{10}H_{18}O$	154.25	Monoterpenoid
Terpinolene	—	—	7.99	0.50	$C_{10}H_{16}$	136.23	Monoterpene
Linalool	—	—	8.64	28.63	$C_{10}H_{18}O$	154.25	Monoterpenoid
cis-Sabinenehydrate	—	—	8.99	0.51	$C_{10}H_{18}O$	154.25	Monoterpenoid
Camphor	7.92	0.28	9.58	8.20	$C_{10}H_{16}O$	152.23	Monoterpenoid
Estragole	9.12	5.26	—	—	$C_{10}H_{12}O$	148.20	Aromatic hydrocarbon
trans-Dihydrocarvone	9.24	0.47	—	—	$C_{10}H_{16}O$	152.23	Monoterpenoid
D-Fenchyl alcohol	9.49	0.18	—	—	$C_{10}H_{18}O$	154.25	Monoterpenoid
Fenchol	9.83	1.78	—	—	$C_{10}H_{18}O$	154.25	Monoterpenoid
1-Borneol	—	—	10.17	15.94	$C_{10}H_{18}O$	154.25	Monoterpenoid
Carvone	10.18	6.12	—	—	$C_{10}H_{14}O$	150.22	Monoterpenoid
l-Carvone	10.26	3.17	—	—	$C_{10}H_{14}O$	150.22	Monoterpenoid
Hexyl butyrate	—	—	10.43	1.56	$C_{10}H_{20}O_2$	172.26	Fatty ester
Cryptone	—	—	10.51	0.58	$C_9H_{14}O$	138.21	Ketonic chelate
α-Terpineol	—	—	10.61	3.25	$C_{10}H_{18}O$	154.25	Monoterpenoid
Anethole	11.23	46.73	—	—	$C_{10}H_{12}O$	148.21	Aromatic hydrocarbon
Bornyl formate	—	—	11.29	0.61	$C_{11}H_{18}O_2$	182.26	Ethyl ester
Linalyl acetate	—	—	11.83	2.18	$C_{12}H_{20}O_2$	196.29	Monoterpenoid
Geraniol acetate	—	—	12.55	1.05	$C_{12}H_{20}O_2$	196.29	Monoterpenoid
Eugenol	12.72	3.75	—	—	$C_{10}H_{12}O_2$	164.20	Carboxylic Acid
Thymol	—	—	12.80	0.68	$C_{10}H_{14}O$	150.28	Monoterpenoid
cis-Jasmone	13.09	0.69	—	—	$C_{11}H_{16}O$	164.24	Fatty Acid
Anisyl acetone	13.17	0.36	—	—	$C_{13}H_{16}O_2$	204.27	Ketonic ether
β-Caryophyllene	13.90	0.22	—	—	$C_{15}H_{24}$	204.35	Sesquiterpene
Germacrene d	15.11	0.22	—	—	$C_{15}H_{24}$	204.35	Sesquiterpene
Eugenyl acetate	15.96	0.91	—	—	$C_{12}H_{14}O_3$	206.24	Carboxylic Acid
β-Farnesene	—	—	16.05	0.81	$C_{15}H_{24}$	204.35	Sesquiterpene
Benzeneacetic acid	17.07	0.17	—	—	$C_8H_8O_2$	136.15	Carboxylic Acid
cis-isoapiole	17.85	0.28	—	—	$C_{12}H_{14}O_4$	222.24	Phenylpropanoids
α-Bisabolol	—	—	20.56	1.57	$C_{15}H_{26}O$	222.37	Sesquiterpenoid
α-ethyl-4,4-dimethoxy-Stilbene	30.91	0.64	—	—	$C_{16}H_{16}O_2$	240.30	Ketonic chelate
1,2-Benzenedicarboxylic acid	—	—	33.67	2.17	$C_8H_6O_4$	166.13	Carboxylic Acid

Table 1: Continued.

Compound	*F. vulgare*		*L. angustifolia*		Formula	Molecular weight (g/mol)	Classification
	RT	Percentage	RT	Percentage			
Monoterpene hydrocarbons		20.43		8.85			
Oxygenated monoterpenes		20.27		83.36			
Sesquiterpene hydrocarbons		0.44		0.81			
Oxygenated sesquiterpenes		0		1.57			
Others		58.8		5.38			
Total		99.94		99.97			
Yield		2.15		2.01			

RT: retention time (min).

Table 2: Contact and fumigant toxicity of the essential oils isolated from *Foeniculum vulgare* and *Lavandula angustifolia* against the adult females of *Tetranychus urticae*.

Bioassay	Essential oil	Results of ANOVA		Results of probit analysis				
		F (df = 4, 10)[a]	P value	24-h LC_{50} with 95% confidence limits[b]	Slope ± SE	χ^2 (df = 3)	Sig.[c]	Toxicity index
Contact toxicity	*F. vulgare*	49.75	1.4446	0.557 (0.445–0.716)	1.181 ± 0.144	3.327	0.351	100.00
	L. angustifolia	33.30	9.3842	0.792 (0.598–1.091)	0.936 ± 0.124	1.620	0.655	0.703
Fumigant toxicity	*F. vulgare*	37.70	0.000005	1.876 (1.786–1.982)	5.335 ± 0.670	3.086	0.379	100.00
	L. angustifolia	18.167	0.0001	1.971 (1.628–2.478)	1.377 ± 0.187	3.975	0.264	0.951

[a]Calculated values are greater than values in F table ($\alpha = 0.05$, F credit = 3.4780). Therefore, they are significant.

[b]% v/v and μL/L air for contact and fumigant toxicity, respectively.

[c]Since the significance level is greater than 0.150, no heterogeneity factor is used in the calculation of confidence limits.

F. vulgare while linalool, 1,8-cineole, 1-borneol and camphor were the main compounds of essential oil of *L. angustifolia*. In the study of Chowdhury et al. [14], anethole (58.5% in seed oil and 51.1% in leaf oil) and limonene (22.9% in leaf oil and 19.6% in seed oil) determined as main components of the seeds and leaves of *F. vulgare*. In the other study, borneol, α-terpinene, linolool, and geranyl proprionate were found as major constituents in the *L. angustifolia* essential oil. The variations could be due to differences in location, elevation, and genetic makeup of the plant or due to an adaptive process to particular ecological conditions [15].

Acaricidal activity of *F. vulgare* and *L. angustifolia* essential oils reported in the present study has been reported earlier by other authors. For example, the essential oils from *F. vulgare* and *L. angustifolia* were toxic against *Varroa destructor* Anderson and Trueman (a major pest of honey bees, *Apis mellifera* L.) [16]. The fumigant toxicity of essential oil extracted from seeds of *F. vulgare* was tested against adult females of *T. urticae* by Amizadeh et al. [17].

Essential oils are characterized by two or three major components at fairly high concentrations (20–70%) compared to others components present in trace amounts. The components include two groups of distinct biosynthetical origin. The main group is composed of terpenes and terpenoids and the other of aromatic and aliphatic constituents, all characterized by low molecular weight [6]. They are made from combinations of several 5-carbon-base (C_5) units called isoprene. The monoterpenes are formed from the coupling of two isoprene units (C_{10}). They are the most representative molecules of the essential oils and allow a great variety of structures. A terpene containing oxygen is called a terpenoid. Hence, monoterpenes are found as two forms; monoterpenes hydrocarbon and oxygenated monoterpenes or monoterpenoids. The sesquiterpenes are formed from the assembly of three isoprene units (C_{15}) [6]. Monoterpenes hydrocarbon, monoterpenoids, and sesquiterpenes are present in our tested study too. Regarding their biological properties, essential oils are complex mixtures of numerous molecules, and their biological effects are the result of a synergism of all components or reflect only those of the main components present at the highest levels according to gas chromatographical analysis [6, 18]. It is suggested that the variability of biological activities of essential oils extracted from different plant species against *T. urticae* could be due to chemical components, differences in their chemical composition, and even in synergic and antagonistic interactions between these components [2, 19, 20].

One of the most attractive features of essential oils is that they are low-risk products and they are relatively well-studied experimentally and clinically because of their use as medicinal products [11]. In terms of ecotoxicology, essential oils are safe to use but not without potential problems. For example, constituents of essential oils are biodegradable, with short half-lives ranging from 30 to 40 h for α-terpineol [21] and in contrast to some synthetic insecticides; no biomagnification has been reported to date [11]. Their short residual half-lives on plants also enhance their compatibility with biological control agents and indigenous natural enemies of pests and reduce risks to honeybees and other foraging pollinators [2].

As cost effective commercial problems, large quantities of plant material must be processed to obtain sufficient quantities of essential oils for commercial-scale tests, situation which also requires breeding these plants in great quantities.

Conflict of Interests

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

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Research Article

Application of Asiatic Honey Bees (*Apis cerana*) and Stingless Bees (*Trigona laeviceps*) as Pollinator Agents of Hot Pepper (*Capsicum annuum* L.) at Local Indonesia Farm System

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In Indonesia, hot pepper (*Capsicum annuum*) is one of the most important spices. Despite the fact that high yield cultivars and fertilizers have been applied to increase the annual production of this spice, local farming is always unable to maintain constant production. Studies to find the explanation of this problem mostly focused on pest attack while possibility of low fruit production due to lack of pollination was neglected. In this study, the effect of pollinator visitation to fruit set and quality was assessed by application of two local domesticated honey bees, Asiatic honey bees (*Apis cerana*) and stingless bees (*Trigona laeviceps*) as potential pollinator agents at hot pepper plantation. This study found that both bees had similar visitation rate while *A. cerana* spend less time in flowers. Visitation by *A. cerana* and *Trigona laeviceps* improved fruit set, fruit production per plant, average fruit weight, and fruit size. This result confirms the importance of cross pollination for hot pepper production and both species could be used as pollination agent for hot pepper. Advantages and disadvantages for each species as pollination agent for local Indonesia farm system are discussed in this paper.

1. Introduction

Hot pepper (*Capsicum annuum* L.) is cultivated and consumed around the world. Its major producers are United States, Mexico, Italy, Japan, India, and Brazil, where this crop has economic importance. Best way to cultivate pepper is in greenhouses which allows production all year round, best management practices, better fruit quality control, lesser or no use of pesticides, earlier harvesting, and superior uniformity of fruits [1]. However, in Indonesia, hot pepper chili usually cultivated at open field where local farmer usually apply best seeds, extensive weed and pest control, and monoculture system in order to obtain high yield. Despite all of these efforts local farmer could not maintain sustained productivity since open field cultivation highly depend on climate condition and ecosystem services, namely, pest control, nutrition cycle, and pollination to produce abundant harvest and good quality fruit [2–5].

Among all available ecosystem services, this study focused on pollination service. Pepper flowers, like those of most cultivated Solanaceae, are pendent from leaf axils, showing a white corolla, five to seven stamens containing 1.0 to 1.5 mg of pollen, and one central style with a round sticky stigma on its top. Anthers are tubular, and dehiscence occurs through lateral opening. Both flower anthesis and anther dehiscence take place in the morning [6]. Although pepper flowers are largely self-pollinated, introducing pollinators could produce beneficial effects on fruit production.

Among pollination agents available in nature, wild insects had been considered as the best pollinator agents and receive huge attention as important component of agriculture systems [7, 8]. In Indonesia, wild pollinator insects have not

yet received notice as important component of agriculture system even though some studies have shown the importance of insects as pollinator for some Indonesian perennial and annual crops [9–13]. Lack of understanding on function of particular insects as pollinator agents combined with common practices of synthetic insecticides, removal of wild plants, and destruction of nesting area through plowing significantly reduced population of wild pollinator [14].

In many intensive plantations, to ensure pollination of crop, domesticated bees usually applied as pollinator agents. In case of pepper pollination, best pollinator agent is bumblebees which carried out "buzz pollination," a mechanism related with behavior of bumblebees in order to release pollen of pepper flowers to pollinate female flowers [15–17]. However, bumblebees are not native species of Indonesia and could potentially cause negative effects on native pollinators and plants [18, 19].

Alternatively, local bee species, such as Asiatic honey bees (*Apis cerana*) and various species of stingless bees (*Trigona laeviceps*), which domesticated by bee farmers for their products (honey, propolis, and wax), may apply as pollinator agents for hot pepper. Previous study showed their possible application as pollinator of tomato flowers, plant with similar flower characteristic with hot pepper flowers [20]. In this study we will evaluate performance of Indonesia domesticated bees as pollination agent of hot pepper and its possible use and concern for application at local farm system.

2. Materials and Methods

2.1. Study Area and Research Materials. The pollination experiment was conducted at local farm in North Bandung, West Java, Indonesia. Average daily temperature of study site was 20–25°C with humidity 70–75%. For this purpose, thirty red chili plants, planted in pots, were arranged in 3 rows of 10 plants each, with two-meter-wide aisle between the rows. Four colonies (≈500 bees per colony) of *Trigona laeviceps* and four colonies of *A. cerana* (≈10,000 bees) were introduced into farm. All colonies were kept in bee hive made from wood and acclimatized for 3 months prior to study.

2.2. Bee Visitation Frequency. Frequency of bee visitation was observed during flowering period by method developed by Klein et al. [12] whereas observation was conducted only at sunny day or 60% cloudy day between 0900 and 1400 (local time). Observation was conducted with interval of 15 minutes for three consecutive days at different plant. Total number of flowers observed for three days was 100 flowers.

2.3. Bee Pollination Efficiency. In this study, 10–20 flowers from each plant (depend on the number of available flowers), that still not bloomed were randomly selected and tagged. Total number of plants used for each experiment group, explained below, was 10. Each group of flower was bagged with mesh nylon bag (diameter 1 mm). Glue was applied at the twig where flowers were located to prevent ant from entering flower.

Bags were kept until fruit production for self-pollinated group. As for bee pollinated group, bags were removed when flower started to bloom. Observation for bee pollination efficiency started from removal of the bag until bee transferred pollen to female flower. After pollination process, flowers were bagged until fruit was produced. This group of treatment was designed as honey bee (HB) and stingless bee (SB) group. Pollination studies of honey bee and stingless bee were conducted in different period. As for control group (NP), bag was not removed from flower until fruit was produced or all flowers has dehiscenced.

Pollination efficiency for each group was measured by

$$\text{Pollination efficiency} = \frac{\text{Total numbers of flowers that produce fruits}}{\text{Total numbers of observed flowers}}. \quad (1)$$

2.4. Fruit Production. Fruit production was measured for every type of pollination. Fruit production was measured by subtracting total number of broken fruits from total fruit produced.

2.5. Fruit Quality. Fruit quality was identified by measuring weight and size of fruit produced from 50 fruits, as soon as they were red in color. The fruits were weighed to the nearest gram and their size was measured to the nearest centimeter.

2.6. Data Analysis. Data was analyzed by statistic program Statistica 8.0 (Statsoft Corp.). Prior to analysis, normality of data was tested. Difference of bee visitation frequency between honey bees and stingless bees was analyzed by *t*-test analysis. Difference of fruit quantity and quality among different pollination types was analyzed by ANOVA and LSD as *post hoc* test. Significant value for both tests was $P < 0.05$.

3. Results and Discussion

3.1. Bee Visitation. We found that visitation rate of honey bee to hot pepper flowers similar to stingless bee (*t-test analysis*, $P > 0.05$) (Figure 1(a)).

Detail observation on visitation pattern of both species showed that honey bee preferred to visit flowers in early morning while stingless bee visited flowers with relatively constant rate (Figure 1(b)). Early foraging bout by honey bees is probably related with availability of fresh pollen and nectar and attractiveness of flower signal. Hot pepper flowers bloom early in the morning, which provides access to fresh pollen and nectar which is located near base of petal [21]. During midday, when most of flowers were dehisced and provided less resources to be harvested by forager honey bees, most of foragers foraged on different flowering plants (Putra, personal observation). The shifting of foraging force of honey bees among food sources was well reported [22–24].

Stingless bees which are smaller and less aggressive preferred late foraging as they usually took long exploratory flight to find rich, suitable, and economic resources [25]. These bees probably exploited remaining pollen and nectar

Table 1: Average fruit production, weight, and fruit size pollinated by local honey bee, stingless bee, and self-pollination ($N = 50$).

Group	*Apis cerana*	*Trigona laeviceps*	Self-pollinated
Fruit Ppoduction per plant	22 ± 3.5^{a}	20 ± 3.5^{a}	17 ± 3.5^{b}
Average fruit weight (g)	12.55 ± 4.17^{a}	11.16 ± 4.99^{a}	9.16 ± 2.99^{b}
Fruit size (cm)	25.16 ± 9.99^{a}	24.78 ± 2.47^{a}	20.58 ± 3.47^{b}

Different letter indicated statistical difference at $P < 0.05$.

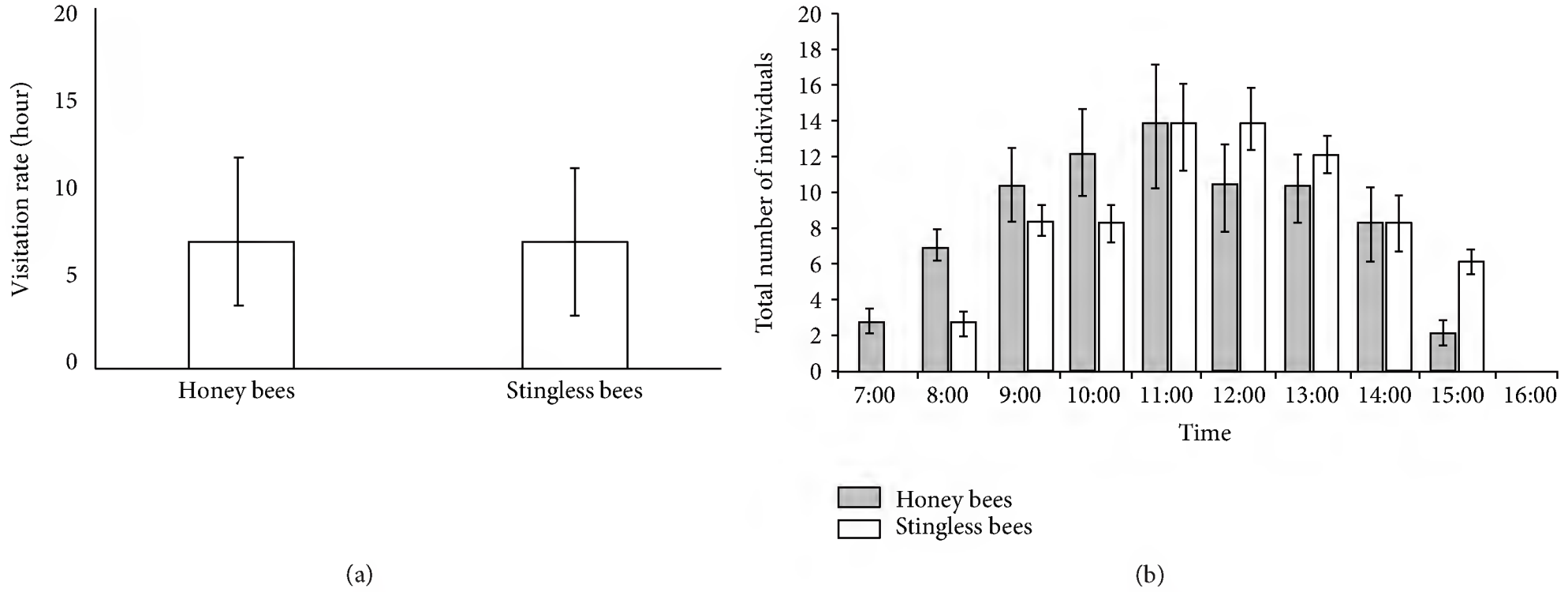

Figure 1: (a) Visitation rate and (b) visitation pattern of honey bees and stingless bees to hot pepper flowers.

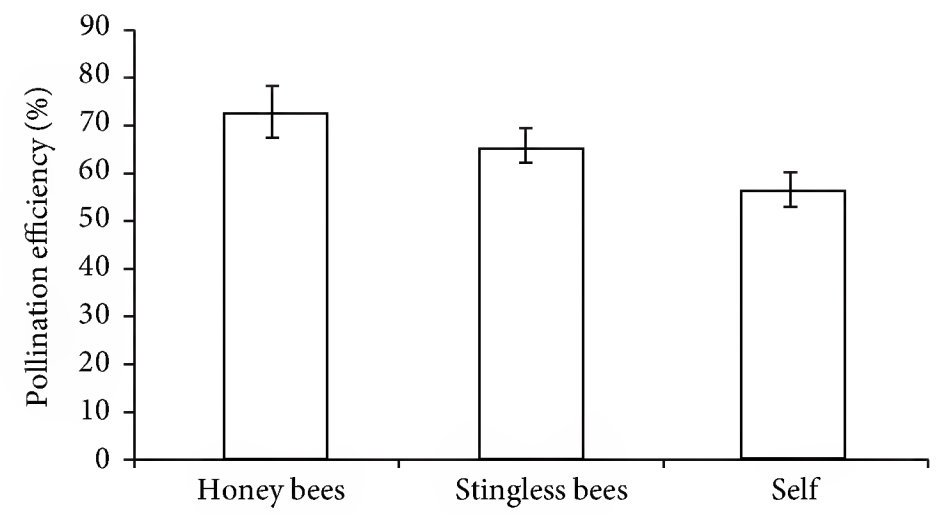

Figure 2: Pollination efficiency of hot pepper by honey bee, stingless bee, and self-pollination.

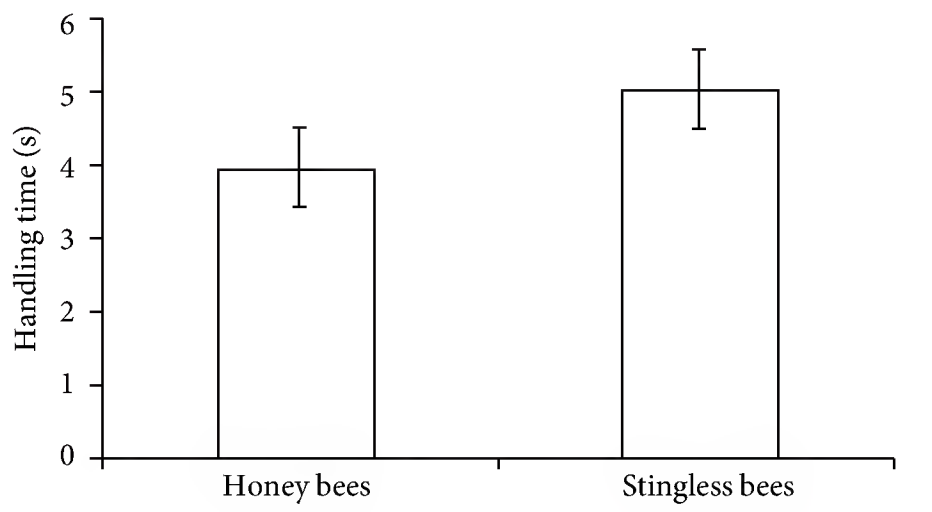

Figure 3: Handling time of honey bee and stingless bee on hot pepper flowers.

which is inaccessible by honey bees due to their bigger size. Stingless bee also tends to specialize on hot pepper flowers, also known as flower constancy, after competition from honey bee was reduced. Flower constancy is widely found on many species of stingless bee [26–28].

3.2. Pollination Efficiency and Fruit Quality. On average, pollination efficiency of honey bee on hot pepper much higher than stingless bee and pollination assisted by both bee species more efficient than wind pollination (*t-test analysis*, $P < 0.05$) (Figure 2).

Total number of fruits produced and quality of hot pepper produces was enhanced significantly by bee pollinated (*One way Anova*, $P < 0.05$). Both bee species provide similar quality of pollination to hot pepper in terms of fruit produce and size of the fruit (Table 1).

This study confirmed that cross pollination improve pollination efficiency and quality of pepper as also reported by de Oliveira Cruz et al. [1], Roldán Serrano and Guerra-Sanz [16], and Al-Abbadi [17].

Based on this data, honey bee and stingless bee have great potential as pollinator insect for hot pepper. Even though both species are known for not showing capability to carry out buzz pollination mechanism [15], they seem to provide some disturbances which improve pollination [29, 30].

Both species provided pollination in different way which is related to handling time. Honey bees seem more economic species because they spend less time in flower, yet produce better pollination success than stingless bees (Figure 3).

Higher pollination success could be caused by their bigger size, which produced more disturbances to flowers, and they tend to visit more fertile flowers. On the other hand, higher flower handling and flower constancy of stingless bee increased the possibility of pollen deposited in stigma.

4. Conclusion

Both species have several advantages and disadvantages when applied on common Indonesia farming.

Asiatic honey bees have higher pollination efficiency, commonly domesticated by local bee farmers, and have wider foraging area which made them suitable candidate as pollinator agent of hot pepper. However, their aggressive and absconding behavior with high nectar and pollen requirement reduce their value as pollinator agent of small hot pepper plantation farm located nearby human residence and/or plantation with unsustain nectar and pollen resources.

On the other hand, stingless bees more likely to be apply as pollinator agent at plantation located nearby human residence without sustain nectar and pollen resources [31, 32]; small foraging area may provide high visitation rate at small and confined agriculture [30, 33]; their lack of functional sting and less aggressive behavior made them highly suitable for pollination of crops cultivated nearby human dwelling [34, 35]. Furthermore, these bees foraging on varied plants [36–38] made them applicable as pollinator for varied types of local crops, even though further studies are needed for possible mismatch (see [35]). However, great concern should be addressed on application of insecticide, a common procedure on Indonesia agriculture system, as stingless bee is highly sensitive to common pesticide applied on local farm (Putra, unpublished data).

Conflict of Interests

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

Authors' Contribution

All authors contributed significantly to this work.

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Research Article

Cryptocephaline Egg Case Provides Incomplete Protection from Generalist Predators (Coleoptera: Chrysomelidae)

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The egg case of *Cryptocephalus rufipes* (Goeze) is described and illustrated. In laboratory trials, eggs of field-collected *C. rufipes* were observed for larval emergence (untreated control) or exposed to two species of generalist predators, *Chrysoperla carnea* (Stephens) or *Xylocoris flavipes* (Reuter) in no-choice experiments. The behaviour of the predators upon contact with the *C. rufipes* eggs was observed. The number of hatching larvae was counted and compared. In the presence of each of the two species of predators, larval emergence was significantly reduced. Eggs that were not protected by an egg case were completely consumed by the predators. *C. rufipes* eggs were therefore incompletely protected from the studied generalist predators. This is the first study showing experimentally the protective function of cryptocephaline egg case.

1. Introduction

Leaf beetles in the subfamilies Cryptocephalinae and Lamprosomatinae cover their eggs with small faecal plates. These faecal plates compose a solid egg case that is completely covering the egg. After hatching, the larva is biting a hole in the egg case, and it is wearing it as a larval case. This larval case is continuously enlarged with larval faeces when the larva is moulting and growing [1–3]. One of the functions of the egg and larval cases is thought to be protection from natural enemies [4, 5]. However, both mammal and insect predators and hymenopterous parasitoids are known to accept cryptocephaline larvae as prey or host, respectively [1, 4–9]. First-instar larvae that are still in their egg cases, but not eggs of *Clytra laeviuscula* and *C. quadripunctata,* were observed to be picked up and transported by ants [10]. However, the larvae of these *Clytra* species are known to live inside the nests of ants [11, 12]; consequently the ants are not expected to prey upon the eggs. While some information on natural enemies of larvae accumulated, nothing seems to be known about natural enemies of cryptocephaline eggs. One of the reasons of this gap in knowledge may be the difficulty to find eggs in nature. Some species attach their egg with the help of a stalk to the host plant [13, 14], where they can be observed in the field. However, the eggs of most species drop to the ground after the female finished building the egg case [3], and then the eggs are difficult to find and observe among the leaf litter. To overcome this problem, in this study adult *Cryptocephalus* (*Burlinius*) *rufipes* (Goeze, 1777) were held in a rearing container, the eggs collected and exposed to two different species of laboratory-reared generalist predators, the common green lacewing *Chrysoperla carnea* (Stephens, 1836) (Neuroptera, Chrysopidae) and the warehouse pirate bug *Xylocoris flavipes* (Reuter, 1875) (Heteroptera, Anthocoridae). Moreover, the egg cases of *C. rufipes* are redescribed and illustrated. *C. rufipes* is widely distributed in Europe, from Portugal to Turkey, and in Northern Africa [15]. In urban areas, it was found feeding on its host plant *Salix purpurea* Linnaeus, 1753, which is planted as a park tree or to form hedges.

2. Materials and Methods

Adult males and females of *C. rufipes* were collected from *Salix purpurea* in Berlin, Friedrichshain (52°52.3272′N, 13°46.5825′E), in June and July, 2014. The adults were kept in 250 mL glass-jars covered with pieces of clothing held with rubber bands at 23 ± 2°C and 60 ± 5% RH. The bottom of the jar was lined with filter paper. A twig of the host

FIGURE 1: Egg of *Cryptocephalus rufipes* (Goeze, 1777), scale = 0.5 mm.

plant *S. purpurea* was placed in a narrow plastic tube (5 × 1.2 cm) filled with water and closed with a plug of paper towel to prevent the water from loss by leakage. The twig was replaced when necessary. Eggs laid by several females were collected daily from the bottom of the jars. The eggs were transferred to Petri-dishes (diameter: 5 cm) lined with paper. To each Petri-dish, five *C. rufipes* eggs and either two adult *X. flavipes*, two larvae of *C. carnea*, or no predators (untreated control) were added. Each treatment had 11 replications. The behaviour of the predators upon first contact with the *C. rufipes*-eggs was observed. In the first three days after adding the predators, the experiments were controlled for survival of the predators. After seven days, eggs were controlled daily for larval emergence. Additionally, five Petri-dishes were prepared with five eggs of *Ephestia kuehniella* each and either two adult *X. flavipes*, two larvae of *C. carnea*, or no predators (untreated control). Laboratory-reared larvae of *C. carnea* and adults of *X. flavipes* were obtained from Biologische Beratung Ltd., Berlin.

The results were analysed with the help of SigmaStat 3.1 software. The number of leaf beetle larvae emerged was subjected to a Kruskal-Wallis One Way Analysis of Variance on Ranks followed by All Pairwise Multiple Comparison Procedures, Dunn's Method, to separate means. Treatments were considered significantly different at the $P = 0.05$ level. Percentage natural mortality data of *C. rufipes* eggs were not corrected for control mortality, because mortality in the untreated control treatment was <5%. The size of 30 eggs, that is, length and width in lateral view, was measured with a measuring ocular mounted on a dissecting microscope.

3. Results

3.1. Field Observations. Beside *C. rufipes*, several other Chrysomelidae were occurring on *Salix purpurea* in June and July, namely *Cryptocephalus* (*Burlinius*) *ocellatus ocellatus* Drapiez, 1819, *C. androgyne* Marseul, 1857, *Phratora vitellinae* (Linnaeus, 1758), and *Clytra laeviuscula* (Ratzeburg, 1837). Moreover, the weevil *Polydrusus* (*Polydrusus*) *picus* (Fabricius, 1792) was found feeding and mating on *S. purpurea*.

3.2. Morphology of C. rufipes Eggs. The eggs of *C. rufipes* are blackish to greyish brown with eight narrow, regular ridges as illustrated in Figure 1. Each individual faecal plate bears a little crest; the regular arrangement of the faecal plates composes the ridges that have little gaps in case the faecal plates do not perfectly touch. The mean size ± SD was 0.797 mm ± 0.057 mm length and 0.532 mm ± 0.033 mm width; median was 0.80 for length and 0.53 for width. Length was ranging from 0.700 to 0.975 mm, width from 0.475 to 0.600 mm. The eggs are elongate oval; the mean length to width ratio was 1.50 mm ± 0.095, ranging from 1.25 to 1.70 (median 1.50).

FIGURE 2: Larva of *Chrysoperla carnea* (Stephens, 1836) uplifting an egg of *Cryptocephalus rufipes* (Goeze, 1777).

FIGURE 3: Adult of *Xylocoris flavipes* (Reuter, 1875) handling an egg of *Cryptocephalus rufipes* (Goeze, 1777).

3.3. Experiments with Eggs of E. kuehniella. The experiments with eggs of *E. kuehniella* resulted in complete predation of these eggs by both *C. carnea* and *X. flavipes*. In the untreated control, 92% of the *E. kuehniella*-eggs emerged.

3.4. Experiments with Eggs of C. rufipes, Behavioural Observations. When encountering the *C. rufipes* eggs, the larvae of *C. carnea* showed the typical prey uplifting behaviour. They fixed the *C. rufipes* eggs and held them in position (Figure 2). *X. flavipes* examined the eggs after contacting it (Figure 3). Within the first three days of the experiment, in all trials one predator consumed the second; consequently, cannibalism occurred.

3.5. Experiments with Eggs of C. rufipes, Larval Hatch. Larvae hatched after 10 to 13 days. The presence of the predators significantly affected the number of hatching *C. rufipes*-larvae from the eggs (Kruskal-Wallis One Way Analysis of Variance on Ranks, $H = 18.473$, DF = 2, $P < 0.001$). In the untreated control, a mean ± SD of 3.23 ± 1.02 larvae hatched. The presence of both *C. carnea* and *X. flavipes* significantly reduced the number of hatching *C. rufipes*-eggs (All Pairwise

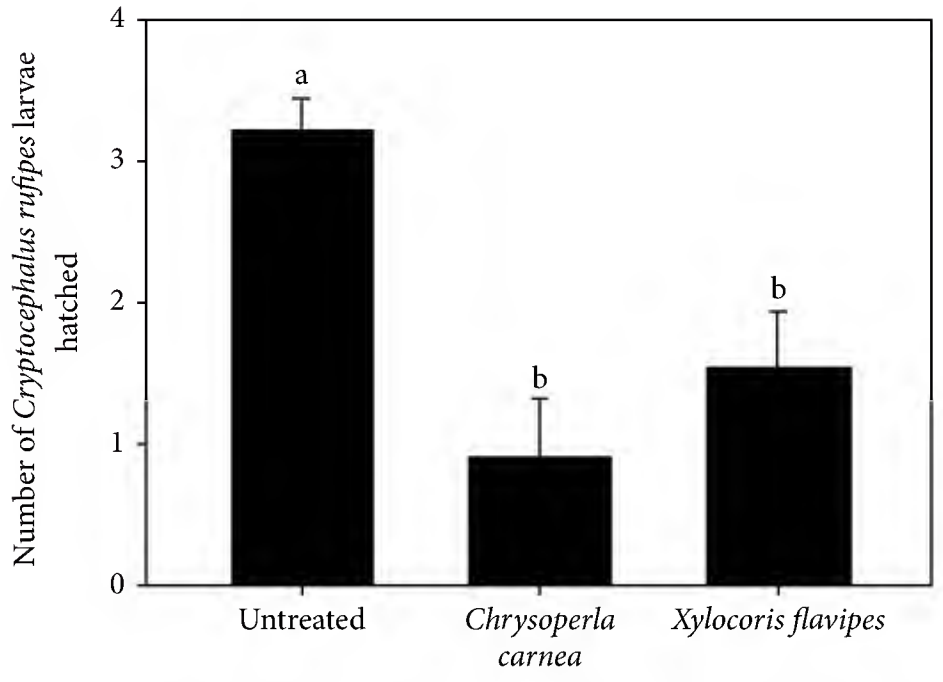

FIGURE 4: Number of *Cryptocephalus rufipes*-larvae hatched out of five eggs exposed to *Chrysoperla carnea* or *Xylocoris flavipes*, or no predators (untreated). Means followed by the same lowercase letter do not differ significantly at $P < 0.05$ (Dunn's Method) ($n = 11$).

Multiple Comparison Procedures, Dunn's Method, $Q = 3.891$ and 2.817, $P < 0.05$). A mean of 0.91 ± 1.38 and 1.55 ± 1.29 hatched in the presence of *C. carnea* and *X. flavipes*, respectively. However, there was no statistical difference in reduction between the two predators (Dunn's Method, $Q = 0.929$, $P > 0.05$) (Figure 4).

4. Discussion

The egg of *C. rufipes* was first described in 1852 [1], under two synonyms, that is, *C. gracilis* Fabricius, 1792, and *C. minutus* Fabricius, 1792. An egg length of 0.75 mm as well as a surface with eight to nine ridges was given. For *C. gracilis*, regular ridges and a blackish green colour were described, and for *C. minutus* irregular ridges and a yellowish green colour. The egg of *C. minutus* was figured [1, Figure 18]: the egg in this figure is less elongate and the ridges are wider compared to the eggs described in the present study. In 1899, again the egg case was described twice under *C. gracilis* and *C. minutus*, possibly mixing information from [1] and own observations [16]. For the eggs described under *C. minutus* [16, page 51], a length ranging from 0.7 to 0.8 was given, data in accordance with those found in the present study. However, the eggs were described as yellowish grey with slightly irregular ridges, contrary to the blackish brown eggs with rather regular ridges described here. For the eggs described under *C. gracilis* [16, page 56], a short note was given stating a length of 0.8 mm, a surface with nine narrow bend carinae and a greyish colour, fitting better to the eggs described here. However, as the determination of species in the subgenus *Burlinius* Lopatin, 1965, requires in many cases the study of aedeagus characters [17], the identity of immatures described in the 19th Century remains sometimes doubtful, and especially the immatures described as *C. minutus* probably belong to another species. Even though the shape of the eggs is variable as indicated by the range of the length to width ratio, they can be, for example, easily distinguished from those of the related synoekous species *C. ocellatus*, which are yellowish brown with blunt ridges.

Concerning natural enemies of *C. rufipes*, reports are available about larval parasitoids [1, 16]. No information was traced on natural enemies of eggs of Cryptocephalinae in general.

In the laboratory trials, feeding could not be directly observed, as both species of predators have piercing-sucking moth parts. However, the behavioural observations indicated the predators identified the *C. rufipes* eggs as prey items. The trials with the *E. kuehniella* eggs showed the ability of the predators to locate the eggs within the experimental arena, and that food was a limiting factor for survival in these no-choice experiments. At least after cannibalism, the predators relied upon the eggs of *C. rufipes* for survival. The analysis of *C. rufipes* hatching showed a significant reduction of larval emergence in the presence of both predators, proving indirectly the predator-induced mortality. However, contrary to the experiments with the eggs of *E. kuehniella*, predation of *C. rufipes* eggs was not complete. This observation suggests egg cases of *C. rufipes* provide a protection against predation by generalist predators, although incomplete.

Larvae of *C. carnea* are known to prey on eggs of various insects [18]. Both adults and larvae of *X. flavipes* are known to prey on insect eggs, including eggs of Chrysomelidae [19]. Both *C. carnea* and related species and anthocorid predators commonly occur in Central Europe. However, they may not frequently encounter *C. rufipes* eggs in the leaf litter because they are typically foraging on leafs. *C. carnea* and *X. flavipes* were used here as model organisms for generalist predators with piercing-sucking mouth parts. Possibly the egg cases provide even better protection against small predators with chewing mouth parts. Natural enemies of cryptocephaline eggs in biotic communities have to be identified and studied in future field studies.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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