

# New records of *Leptopilina*, *Ganaspis*, and *Asobara* species associated with *Drosophila suzukii* in North America, including detections of *L. japonica* and *G. brasiliensis*

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## Abstract

We report the presence of two Asian species of larval parasitoids of spotted wing *Drosophila*, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), in northwestern North America. *Leptopilina japonica* Novkovic & Kimura and *Ganaspis brasiliensis* (Ihering) (Hymenoptera: Figitidae) were found foraging near and emerging from fruits infested by *D. suzukii* at several locations across coastal British Columbia, Canada in the summer and fall of 2019. While *G. brasiliensis* was found in British Columbia for the first time in 2019, re-inspection of previously collected specimens suggests that *L. japonica* has been present since at least 2016. Additionally, we found a species of *Asobara* associated with *D. suzukii* in British Columbia that is possibly *Asobara rufescens* (Förster) (known only from the Palearctic Region) based on COI DNA barcode data. These findings add to the list of cases documenting adventive es-

establishment of candidate classical biological control agents outside of their native ranges. The findings also illustrate the need for revisiting species concepts within *Asobara*, as well as host and geographic distribution data due to cryptic and/or misidentified species.

### Keywords

adventive establishment, Braconidae, classical biological control, Drosophilidae, Figitidae, Pteromalidae, spotted wing *Drosophila*

## Introduction

Evidence is accumulating that increasing human activities are responsible for the redistribution of not only invasive pest insects but also many of their natural enemies (Roy et al. 2011; Mason et al. 2017; Weber et al. 2017). In the past few decades, there have been numerous cases where the arrival of invasive insect pests in their invaded range has been followed by the detection of closely associated parasitoids from their native range, which are assumed to be adventive; that is, accidentally introduced (Frewin et al. 2010; Mason et al. 2011, 2017; Talamas et al. 2015; Weber et al. 2017; Ganjisaffar et al. 2018; Stahl et al. 2019). In some cases, at the time of detection, these adventive parasitoids were under consideration for importation and intentional introduction against their pest hosts as classical biological control agents (Mason et al. 2011; Talamas et al. 2015; Abram et al. 2019). These new distribution records are made possible as a result of close collaboration between the biological control and taxonomic communities (Rosen 1986; Rosen and DeBach 1973; Talamas et al. 2015; Buffington et al. 2019).

Spotted wing *Drosophila*, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), is an invasive pest in North America, South America, and Europe that lays its eggs and completes its larval development inside the fruit of a large variety of crop and non-crop host plants (Lee et al. 2011; Asplen et al. 2015; Kenis et al. 2016). A few species of resident pupal parasitoids, including *Pachycrepoideus vindemmiae* Rondani (Hymenoptera: Pteromalidae), were found attacking *D. suzukii* in these invaded areas but parasitism levels were generally low (Lee et al. 2019). Larval parasitism of *D. suzukii* in invaded areas appears to be particularly uncommon, probably due to the inability of most genotypes of native larval parasitoids to overcome the immune defenses of *D. suzukii* (Lee et al. 2019; Kacsoh and Schlenke 2012; Poyet et al. 2013). Thus, soon after the introduction and spread of *D. suzukii* in invaded areas, exploration for potential classical biological control agents in China, South Korea, and Japan began with the goal of identifying parasitoid species that could be evaluated as potential classical biological control agents (Daane et al. 2016; Girod et al. 2018a; Giorgini et al. 2019). These surveys found several parasitoid species attacking *D. suzukii* larvae and pupae, but two larval parasitoids were responsible for the greatest levels of parasitism: *Ganaspis brasiliensis* (Ihering) and *Leptopilina japonica* Novkovic & Kimura (Hymenoptera: Figitidae). These two species both parasitize early-instar larvae of *D. suzukii* inside fresh fruit (Wang et al. 2018),

although *L. japonica* will also attack hosts in other substrates (e.g., banana traps) (Daane et al. 2016; Giorgini et al. 2019). *Leptopilina japonica* is known to attack Drosophilidae other than *D. suzukii* in the field in Asia (Novković et al. 2011). *Ganaspis brasiliensis* has only been reared from fresh fruits infested by *D. suzukii* and other closely related, microsympatric frugivorous Drosophilidae and thus appears to have a more restricted host range than *L. japonica* (Daane et al. 2016; Girod et al. 2018a; Giorgini et al. 2019). The host specificity of *G. brasiliensis* may also vary significantly among genetic lineages (Nomano et al. 2017; Girod et al. 2018b). Recently, *G. brasiliensis* was reared from sentinel traps containing larvae and pupae of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) in Mexico (Gonzales-Cabrera et al. 2020), representing the first time this species has been detected in continental North America.

Exploration for biological control agents of *D. suzukii* has also led to the discovery of described and several undescribed species of *Asobara* (Hymenoptera: Braconidae) attacking *D. suzukii* in Asia (Nomano et al. 2015; Guerrieri et al. 2016). *Asobara japonica* Belokobylskij was initially considered as a potential classical biological control agent of *D. suzukii* but was subsequently excluded from consideration due to its relatively broad host range (Daane et al. 2016; Girod et al. 2018b). Both Asian and European lines of another species in the same genus, *Asobara tabida* Nees, failed to develop on *D. suzukii* in laboratory experiments (Chabert et al. 2012; Nomano et al. 2015). North American *Asobara* species are not presently known to parasitize *D. suzukii*, although one species (putatively *A. tabida*) has been observed in a possible association with *D. suzukii* (Thistlewood et al. 2013).

In this article, we report the unexpected presence of both *L. japonica* and *G. brasiliensis* in northwest North America, representing the first time they have been detected in this area. These are the first records of *G. brasiliensis* in North America outside of Mexico, and the first records of *L. japonica* outside of its presumed native range of Asia. We also report one species of *Asobara* (Hymenoptera: Braconidae), as well as *Pachycrepoideus vindemmia* (Hymenoptera: Pteromalidae), in probable association with *D. suzukii*.

## Materials and methods

### Preliminary parasitoid collections

On July 15, 2019, we first observed numerous parasitoids walking on and flying in the vicinity of raspberry (*Rubus idaeus* ‘Rudi’) fruits in an experimental plot heavily infested by *D. suzukii* in Agassiz, British Columbia, Canada (GPS: 49°14'34.8"N, 121°45'18.0"W). Remarkably, we collected > 100 parasitoid specimens by mouth aspirator in less than 45 min of effort by two individuals (PKA, personal observations). We also collected 20 raspberry fruits presumed to be infested by *D. suzukii*, which were divided between two ventilated plastic containers (0.25 L) with filter paper to observe whether any parasitoids successfully emerged. A subset of field-collected parasitoid individuals were exposed to 2<sup>nd</sup> and 3<sup>rd</sup>-instar *D. suzukii* larvae (approx. 50 per vial) in

two *Drosophila* rearing vials containing artificial diet (Formula 4–24 medium, Carolina Biological Supply Co. Burlington, NC) to informally test whether or not they were capable of successfully parasitizing *D. suzukii* larvae. Parasitoids emerged both from field-collected *D. suzukii*-infested raspberries and lab-reared *D. suzukii* larvae in artificial diet exposed to field-collected parasitoid adults (PKA, personal observations). This was surprising, given the known low developmental success of North American larval parasitoids attacking *D. suzukii* (Lee et al. 2019). This observation motivated an attempt to obtain a preliminary species-level identification. A 683 bp region of the cytochrome oxidase I (COI) gene of one aspirator-collected parasitoid specimen was bar-coded and compared against existing records in GenBank using a nucleotide BLAST (NCBI, 2019). This sequence showed 98.67 % identity with GenBank Accession AB546875.1, which is a sequence of *L. japonica* from Japan (Novkovic et al. 2016). Specimens from the same date and location that were collected by mouth aspirator (n = 24), emerged from raspberry fruit (n = 3), and that emerged from lab-reared *D. suzukii* larvae (n = 6), were all identified as *L. japonica* (by MLB) based on morphology (see *Identification of parasitoid specimens* below for details on morphological determinations). This initial find prompted a re-examination of parasitoid specimens (n = 14) emerging from 60 *D. suzukii*-infested strawberry (*Fragaria × ananassa*) fruits (total n = 375 emerging *D. suzukii*) collected near Langley, British Columbia in two commercial fields (GPS: 49°07'34.7"N, 122°32'45.2"W and 49°08'07.8"N, 122°33'42.1"W) between September 20 and 27, 2016. Based on morphology, the parasitoid specimens were also confirmed to be *L. japonica*, suggesting that this species has been present in British Columbia since at least 2016.

### Parasitoid collections throughout southwestern British Columbia

Motivated by the unexpected detection of *L. japonica* in British Columbia, we next conducted additional collections in 2019 to determine how widespread in coastal British Columbia *L. japonica* might be, and whether any other native or exotic species of parasitoids might also be present. From August to October 2019, parasitoid specimens were obtained from 54 opportunistic collection samples from 23 different sites in coastal British Columbia (the lower mainland and Vancouver Island) (Fig. 1). Parasitoid specimens (n = 525) were collected from plants and areas known to be infested by *D. suzukii* with one of three sampling methods: (1) mouth aspiration of parasitoids seen flying or resting on the foliage or fruits of Himalayan blackberry, *Rubus armeniacus* (n = 34 samples) or cultivated blackberry, *Rubus fruticosus* ‘Triple Crown’ (n = 2 samples); (2) collection of *R. armeniacus* fruits (approx. 20–60 berries per sample) into ventilated plastic rearing containers (volume: 0.5 or 1.0 L) lined with filter paper to rear out parasitoids and host Drosophilidae in the laboratory (16:8 hour light:dark, 24 ± 1 °C) (n = 14); (3) separation of parasitoid by-catch from plastic traps baited with apple cider vinegar placed in hedgerows on a university experimental farm to collect *D. suzukii* (n = 2); and (4) collection of *R. armeniacus* berries followed by exposure outside

in a plastic trap for 6 days, and then rearing out insects in glass jars in the laboratory ( $n = 2$ ). Collections from *R. armeniacus* were in suburban or natural areas along roadways or in parks. For a subset of 14 *R. armeniacus* fruit collections from which at least one parasitoid emerged, emerging Drosophilidae ( $n = 1,637$ ) were also counted and identified. All identifiable emerged Drosophilidae were either *D. suzukii* (85.8 %) or *D. melanogaster* (0.6 %). Remaining flies (13.6 %) could not be identified conclusively due to deterioration or mold in containers. Full information on collection sites, dates, and methods, and insect emergence if applicable, can be found in the Suppl. material 1. Due to the opportunistic nature of our sampling in space and time, and unknown levels of parasitoid and host mortality in rearing, we did not calculate or present percent parasitism levels. All collected parasitoids were preserved in 95 % EtOH for subsequent morphological and molecular identification.

### Identification of parasitoid specimens

Specimens from both initial and more extensive collections in British Columbia were sent to MLB after initial COI barcoding (by SJP and CC) yielded results consistent with barcode sequences on GenBank under the name *L. japonica* (see above). Upon further examination, it was determined that not only was *L. japonica* present in the samples but so too were *G. brasiliensis*, an undetermined species of *Asobara*, and *P. vindemmiae* (Figs 2–7). Specimens of *L. japonica* and *G. brasiliensis* were vacuum dried and card mounted; the *Asobara* and *Pachycrepoideus* specimens were prepared using HMDS (Heraty and Hawks 1998) and point mounted. Specimens are deposited in the insect collection of the National Museum of Natural History (USNM; Smithsonian Institution, Washington DC) and the Canadian National Collection of Insects, Arachnids, and Nematodes (Ottawa, Ontario, Canada).

The morphology-based research in Novković et al. (2011), Buffington and Forshage (2016) and Lue et al. (2016), as well as specimens in the USNM, were used to confirm the identities of the Figitidae. While this research was being conducted, it became clear that specimens of *Leptopilina* submitted to MLB for identification in 2016 were misidentified as *Leptopilina heterotoma* (Thompson). At that time, the USNM lacked specimens of *L. japonica*, making a direct comparison impossible. Recently, the USNM has received voucher specimens from the original paper describing *L. japonica*, as well as additional material from China and Korea. This made direct comparisons possible, and the identity of the 2016 specimens was corrected.

In terms of Figitidae attacking *D. suzukii*, *L. japonica* and *G. brasiliensis* can be difficult to distinguish (Figs 2, 3). Careful examination of the posterior aspect of the metapleuron reveals a complete patch of setae in *G. brasiliensis* (Fig. 4a), while the same location in *L. japonica* is glabrous (Fig. 5a). Additionally, the metasoma setal hair ring is complete in *G. brasiliensis* (Fig. 4b) and dorsally incomplete in *L. japonica* (Fig. 5b). Lastly, the metapleuron, in lateral view, is simple in *G. brasiliensis* (Fig. 4c); in *L. japonica*, a longitudinal carina transverses the middle of the metapleuron (Fig. 5c).

Specimens of *Asobara*, *Pachycrepoideus*, and Figitidae are relatively easy to differentiate from one another given their overall dissimilar shape and color (Figs 6, 7).

The single Pteromalidae specimen was identified and confirmed using Bouček and Heydon (1997) and by comparison with authoritatively identified specimens in the USNM. The specimens of *Asobara* were sight identified to genus; Wharton (1980), Vet et al. (1984), and Nomano et al. (2015) were consulted in an effort to identify the specimens to species.

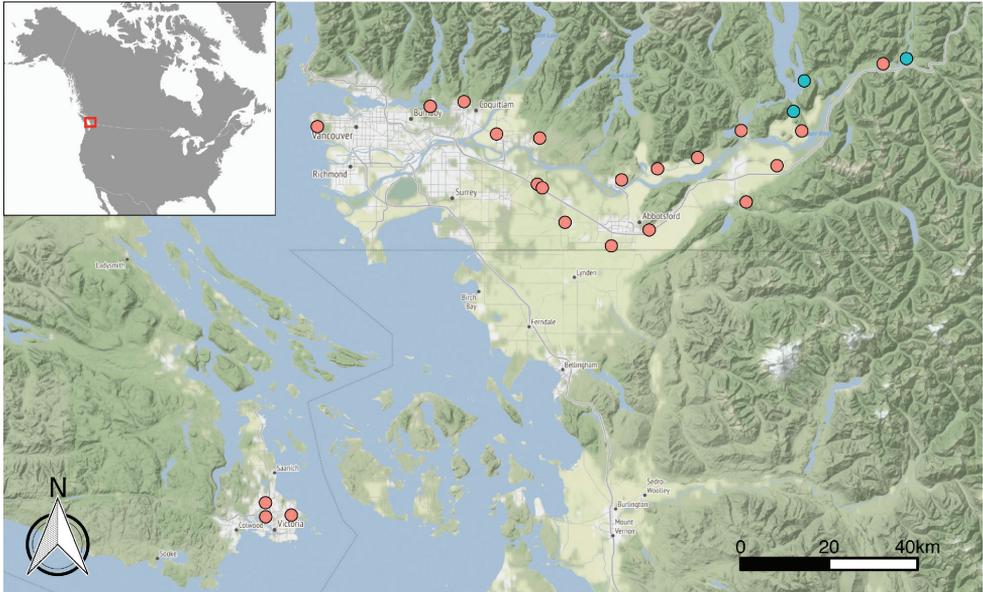
A subset of the Figitidae and Braconidae specimens were sequenced for the COI ‘barcode’ region; as only one *Pachycrepoideus* was collected, no barcode was generated for this species. DNA was extracted using the QIAamp DNA Micro Kit (Qiagen). PCR amplifications of COI were carried out using LCO1490 (Folmer et al. 1994) and C1-N-2191 (Simon et al. 1994) with a Bio-Rad T100 thermal cycler under the following cycling conditions: initial denaturation for 7 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 50 °C, 45 s at 72 °C, and a final extension for 1.5 min at 72 °C. PCR products were cleaned with ExoSAP-IT (Affymetrix), sequenced with BigDye v.3.1 (Life Technologies) and run on an ABI 3730xl automated DNA sequencer (Applied Biosystems). Contigs were assembled and edited in Geneious Prime 2020.04 (Biomatters). Sequences used in the study have been deposited in GenBank (Accession nos. MT559416–MT559426).

## Results and discussion

### Figitidae

*Leptopilina japonica* was the most common parasitoid species found in our collections, representing 95.8 % (503/525) of all parasitoids we collected and identified in British Columbia in 2019. This species was present across the sampled area (Fig. 1) and was collected with all three sampling methods (traps, aspirator collections, rearing from infested fruit). *Ganaspis brasiliensis* was the next most common species (2.7 % of all specimens) and was present at three sites, which were all in the eastern part of the sampled geographic range (Fig. 1). This species was collected both with aspirators while foraging on berries and emerging from *D. suzukii*-infested berries. Somewhat surprisingly, no native *Leptopilina* spp. were found in our samples in 2019.

Results from DNA barcoding of a small subset of Figitidae specimens was consistent with all morphologically-based identifications. COI sequences from *L. japonica* showed high similarity to specimens previously collected in China and Japan (> 98 % identity). All four of the *G. brasiliensis* specimens from British Columbia that we bar-coded clustered within the “G1” clade (100 % identity), which has previously been found in South Korea, China, and Japan (Nomano et al. 2017; Giorgini et al. 2019) and appears to be the most host-specific lineage of *G. brasiliensis* (Nomano et al. 2017; Girod et al. 2018b). Further studies are needed to determine the levels of genetic diversity of *L. japonica* and *G. brasiliensis* in North America and potential source populations of these parasitoids in Asia.



**Figure 1.** Map of sites where *Leptopilina japonica* (red circles), or both *L. japonica* and *Ganaspis brasiliensis* (green circles) were found in British Columbia, Canada in 2016 and 2019. The red box in the inset shows where the mapped area is situated in North America. Map tiles by Stamen Design, under CC BY 3.0. Data by OpenStreetMap, under ODbL.

We emphasize that because our sampling took place within a restricted time window and set of habitats, our data are likely not representative of the two adventive figitid parasitoid species' relative abundances or impact. They simply demonstrate that these two species are present in the south coastal region of British Columbia. More widespread, season-long surveys in several habitats are needed to determine the relative abundance and distribution of each species. Similarly, repeated fruit collections containing known life stage distributions of hosts, coupled with population modelling approaches such as stage-structured matrix models (e.g., Wiman et al. 2014, 2016), will be necessary to estimate the population-level impact of these adventive exotic parasitoids attacking *D. suzukii* – and possibly other, non-target Drosophilidae – in North America.

## Braconidae

One species of *Asobara* (1.5 % of all parasitoids in collections) was collected with aspirators from berries (n = 3) and reared from *D. suzukii*-infested blackberries (n = 5) from two sites. Although nearly all of the Drosophilidae emerging from these collections were *D. suzukii*, we cannot exclude the possibility that the five *Asobara* that emerged from berries could have been associated with *D. melanogaster*, which was present at very low levels in our samples (see above). Exposure of three female *Asobara* to lab-reared *D. suzukii* larvae in artificial diet (see methods in “Preliminary parasitoid



**Figures 2–7.** *Ganaspis brasiliensis* (2, 4); *Leptopilina japonica* (3, 5); *Pachycrepoideus vindemmiae* (6); *Asobara* sp. (7).

collections” above) only yielded a single male offspring (PKA, personal observations). While the field collections and single lab observation provides a proof-of-concept that this *Asobara* species can attack and develop in *D. suzukii*, at this time the association of this *Asobara* species with *D. suzukii* should be considered tentative. As far as is known, species of *Asobara* are solitary koinobiont endoparasitoids of cyclorrhaphous flies, mostly Drosophilidae (Wharton 1997, Yu et al. 2016).

Two species of *Asobara*, *A. tabida* (Nees) and *A. fungicola* (Ashmead), are known from the Nearctic Region, and both species have been reported from British Columbia (Yu et al. 2016). One of us (RRK) initially identified the *Asobara* we found associated with *D. suzukii* in British Columbia as *A. tabida* using Wharton (1980), but *A. tabida* and *A. fungicola* are the only species included in the key. Several species of *Asobara*, including *A. tabida* and *A. fungicola*, occur in multiple zoogeographic regions (Yu et al.

2016). Therefore, it is possible that other *Asobara* species morphologically similar to *A. tabida* and *A. fungicola* occur in the Nearctic Region but are commonly misidentified as the aforementioned species. For example, *Asobara rufescens* (Förster) and *A. tabida* are extremely similar morphologically; Vet et al. (1984) considered them sibling species and identified subtle differences in color, shape of antennal flagellomeres 1 and 2, tarsal claw length, and the basitarsus. *Asobara rufescens* (Förster, 1863) is currently known only from the Palearctic Region but has been considered a junior synonym of *A. tabida* (Nees, 1834), which occurs in the Nearctic, Palearctic, Oriental, and Oceanic regions. Thus, if *A. rufescens* occurs in the Nearctic Region it could be easily misidentified as *A. tabida*, especially when using identification tools that were based on region or published during the period of time when *A. rufescens* was a junior synonym of *A. tabida*. Recent efforts to accurately identify species of *Asobara* have relied on COI DNA barcoding along with authoritative identifications based on morphological features (Nomano et al. 2015, Guerrieri et al. 2016).

We obtained a 658-bp fragment of the COI DNA barcoding region from one of the specimens RRK tentatively identified as *A. tabida*. A GenBank nucleotide BLAST search (NCBI 2019) using the 658-bp fragment, as well as a search of all COI barcode records using the Identification System in the Barcode of Life Database (BOLD; Ratnasingham and Hebert 2007), yielded 53 COI sequences we consider conspecific with our *Asobara* specimen. Of the 41 sequences from GenBank, 39 had 98.79–100 % identity (79–100 % query coverage) with the *Asobara* COI sequence obtained in this research; percent identities for the two most dissimilar sequences that we still consider conspecific were 97.24 % and 97.26 % (100 % and 93 % query coverage, respectively). The 41 sequences from GenBank were mostly from specimens identified as *A. rufescens* ( $n = 36$ ) but also included some identified only as Braconidae or Hymenoptera ( $n = 5$ ). The sequences in BOLD consisted of the 41 recovered via GenBank and an additional 12 sequences we consider conspecific with our *Asobara* species. Of the 53 sequences recovered, 51 have sequence similarity ranging from 99.18–100 %, and two have sequence similarity ranging from 97.19–97.22 %; all are in BOLD under the name *A. rufescens*. One sequence in both GenBank and BOLD under the name *A. rufescens* (GenBank: KT604569.1) appeared to have quality control issues and was not considered in this research. The most similar sequence in GenBank and BOLD that we do not consider conspecific was from a specimen identified as *A. tabida*, with 93.46 % identity (99 % query coverage) in GenBank and 93.36 % similarity in BOLD relative to our *Asobara* sequence.

Sequences in BOLD/GenBank identified as *A. rufescens* were likely determined as such based solely on their high similarity to sequences already in those databases from specimens identified as *A. rufescens* using morphological features. Notably, COI sequences from putative *A. rufescens* specimens from Japan (Nomano et al. 2015; GenBank: AB920758.1, AB920759.1, AB920763.1, AB920762.1), identified by M. Kimura using the morphological features from Vet et al. (1984), are likely the basis for all subsequent COI sequence identifications in BOLD/GenBank. If the specimens from Japan are accurately identified as *A. rufescens*, a specimen of *A. rufescens* collected

in Puslinch, Ontario, Canada on August 14, 2008 (Hebert et al. 2016; GenBank: KR884537.1) would be the first record of *A. rufescens* in the Western Hemisphere. Further, collection data for all sequences in BOLD/GenBank putatively conspecific with M. Kimura's *A. rufescens* reflect that this species was collected in Canada every year from 2008–2019 (except 2011) with occurrences ranging from Forillon National Park, Quebec (Hebert et al. 2016; GenBank: KR420364.1) to Elk Island National Park, Alberta (Hebert et al. 2016; GenBank: KR793444.1). Thus, *A. rufescens* has possibly occurred in North America since at least 2008, the year *D. suzukii* was first detected on the continent (Asplen et al. 2015), and ranges from east to west across nearly all of Canada. Thistlewood et al. (2013) reported specimens of *Asobara* reared from fermenting banana that was artificially infested with *D. suzukii* and then placed in the field in British Columbia, which they considered likely to be *A. tabida*. The *Asobara* specimens reported in Thistlewood et al. (2013) might be conspecific with our *Asobara* species from British Columbia. If so, it is possible that our *Asobara* species (possibly *A. rufescens*) has been associated with *D. suzukii* since at least 2011; however, this is uncertain regardless of *Asobara* species identity because the banana baits were infested with Drosophilidae other than *D. suzukii* after being placed in the field.

Kimura's identification of *A. rufescens* from Japan, based on the morphological features identified in Vet et al. (1984) for differentiating *A. rufescens* and *A. tabida* (Noman et al. 2015, M. Kimura personal communication), should be considered tentative. Features used by Vet et al. (1984) lack enough detail to reliably differentiate species of *Asobara* that are, perhaps, morphologically cryptic (R. Kula personal observation). The features used were differences, "in the shape of the 3<sup>rd</sup> and 4<sup>th</sup> antennal segment... and in the relative length of the claws," as well as unspecified differences "in the colour" and "in the basitarsus of the hind legs" (Vet et al. 1984). These features might reflect differences between *A. rufescens* and *A. tabida*, but accurate interpretation is difficult because the color differences were not illustrated or specified, the basitarsus difference was not elaborated or quantified, and the shape and length differences in the flagellomeres and tarsal claws, respectively, were not quantified. Line drawings were used to illustrate the relevant portions of the antenna and tarsus for specimens Vet et al. (1984) considered *A. rufescens* and *A. tabida*, as well as an *A. rufescens* × *A. tabida* hybrid; thus, identification requires interpreting subtle differences in the line drawings by eye and without consideration for potential intraspecific variation. Thus, the features identified in Vet et al. (1984) require additional assessment to determine their utility.

Beyond morphological features, Vet et al. (1984) discerned via olfactometer tests that what they initially considered *A. tabida* actually consisted of two species attracted to different host habitats. One of the species was attracted to the odor of fermenting fruit, while the other species was attracted to the odor of decaying leaves, thus resulting in a pre-mating reproductive barrier. The authors discerned, through comparison of their *Asobara* specimens with the holotype of *A. rufescens* for aforementioned morphological features, that the specimens attracted to decaying leaves were *A. rufescens*. Thus, the authors regarded *A. tabida* as a parasitoid of frugivorous Drosophilidae and *A. rufescens* as a parasitoid of saprophagous Drosophilidae. Mitsui et al. (2007) reported

*A. tabida* and *A. rufescens* from Drosophilidae, including the former from *D. suzukii*, infesting clumps of banana placed at field sites; they also reported *A. rufescens* from *Scaptomyza pallida* (Zetterstedt) in decaying leaves. However, Chabert et al. (2012) found that *A. tabida* rarely oviposits into *D. suzukii*. Other research has shown, based on analysis of COI DNA barcoding data, that other *Asobara* specimens perceived as conspecific are apparently complexes of morphologically similar species (Nomano et al. 2015, Guerrieri et al. 2016) with different patterns of host use. Thus, additional research is needed to discern the limits of species in *Asobara*, as well as patterns of host use and factors that affect those patterns.

The COI sequence from our *Asobara* species from British Columbia was also very similar to COI sequences from putative *A. rufescens* collected in Germany (BOLD: 99.28–99.53 % similarity; Matthias et al. 2016) and Japan (GenBank: 99.20–99.24 % identity, 94–100 % query coverage; BOLD: 99.18–99.23 % similarity; Nomano et al. 2015). *Asobara rufescens* has been collected in the Palearctic Region from Japan to the Netherlands (Yu et al. 2016); thus, if the identifications in GenBank and BOLD are accurate, *A. rufescens* is distributed broadly across the entire Holarctic Region. Conversely, while *A. tabida* has been reported across the entire Palearctic Region, from Japan and Russia (i.e., Sakhalin Oblast) west to Ireland, it has been reported infrequently in North America (Wharton 1980, Kraaijeveld and van der Wel 1994, Hoang 2002). Interestingly, there are no COI DNA barcode sequences in either GenBank or BOLD from specimens of *A. tabida* in North America. This raises the possibility that specimens in North America identified previously as *A. tabida* are actually *A. rufescens*. The results reported herein demonstrate the need for discerning species complexes within *Asobara* overall and its implications for interpreting patterns of host use. It would also be useful to sequence nuclear genes in *Asobara*, as some of the confusion in resolving *A. tabida* and *A. rufescens* may be due to hybridization and mitochondrial introgression.

## Pteromalidae

Only a single specimen of *P. vindemmiae* was found in our collections. *Pachycrepoideus vindemmiae* is an idiobiont ectoparasitoid recorded as a primary parasitoid of Diptera, Lepidoptera, Hemiptera, and Hymenoptera. Diptera account for most records with 13 families, 33 genera, and 55 species recorded (Noyes 2019). As a facultative hyperparasitoid, hosts include Diptera (Sarcophagidae, Tachinidae) and Hymenoptera (Braconidae, Diapriidae, Encyrtidae, Figitidae, Eulophidae, Pteromalidae). Various factors influence the degree of primary vs. secondary parasitism in *Drosophila* systems (Bezerra Da Silva et al. 2019, Chen et al. 2015, Goubault et al. 2003, Rossi Stacconi et al. 2015, Wang et al. 2016). Related to the work herein, *P. vindemmiae* is known as a parasitoid of *D. melanogaster*, *A. tabida* and *L. heterotoma* (Noyes 2019, Philips 1993, Van Alphen and Thunnissen 1982), and also parasitizes conspecifics (Chen et al. 2015). Future studies could investigate whether *P. vindemmiae* could act as a facultative hyperparasitoid of *L. japonica* and *G. brasiliensis* in North America.

## Conclusions

Our findings here add to the growing list of recent high-profile invasive insect pests whose closely associated natural enemies have established adventive populations on new continents (Frewin et al. 2010; Talamas et al. 2015; Mason et al. 2017; Weber et al. 2017; Ganjisaffar et al. 2018; Stahl et al. 2019). Given that we found *L. japonica* to be remarkably common and widespread in our study area, it is quite possible that this species has also established in other areas outside of Asia but has not been detected yet. *Ganaspis brasiliensis* was much less common and widespread in our collections, and until more extensive sampling throughout the entire season is done, we cannot conclude whether or not it is well established in British Columbia. The *Asobara* species (potentially *A. rufescens*) we collected in probable association with *D. suzukii* also merits further investigation, both in terms of its systematics in relation to other *Asobara* worldwide and the extent of its potential impact on *D. suzukii*. We encourage researchers across North America, South America, and Europe to re-evaluate whether these parasitoids may be attacking *D. suzukii* in other invaded areas.

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## Supplementary material I

### Collection information for specimens reported in: New records of *Leptopilina*, *Ganaspis*, and *Asobara* species associated with *Drosophila suzukii* in North America, including detections of *L. japonica* and *G. brasiliensis*

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Data type: raw data

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# Sampling of parasitoid Hymenoptera: influence of the height on the ground

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## Abstract

Parasitoid hymenopterans are a highly diverse group of insects; therefore, the choice of an adequate sampling method becomes important to achieve a representative species richness of a site. The aim of this work is to evaluate the size and diversity of parasitoids in relation to the height of the Malaise trap placement above the ground of a low deciduous forest from Yucatan, Mexico. Parasitoids were collected from September to October 2015, using three Malaise traps at ground level and other three located right above the others, leaving no space between them, at a height of 1.5 m. The collected specimens were identified at family level. A total of 4083 parasitoids belonging to 31 families were collected, representing 93% of the sample's completeness, according to Jack 1 estimator; with differences in richness and abundance between trap heights according to rarefaction and fixed effects multifactorial ANOVA, respectively. Bethyilidae, Braconidae and Ichneumonidae were the most abundant families. Besides, when analyzing the differences of each family by separate, there were significant results for Bethyilidae, Diapriidae and Ichneumonidae with more individuals in the traps at ground level than in the raised ones. In a further analysis, the effect of body size on the capture height was observed. The specimens of larger size belonging to the families Bethyilidae, Sphecidae, Sclerogibbidae and Evaniidae were more collected at ground level, on the other hand, the larger sized Ichneumonidae were collected at raised level.

## Keywords

diversity, families, malaise, neotropics, size

## Introduction

The choice of an adequate sampling method is indispensable to ensure representativeness of the obtained samples and to infer accurate conclusions regarding the diversity of a site. The later becomes particularly important when collecting insects, since the use of different kind of traps are very frequent to quantify them in view of their size and flight capacity (Mazón and Bordera 2008; Aguiar and Santos 2010). Among insects, parasitoids represent one of the most important biological strategies due to their role as population control of other insects, mostly phytophagous. These insects develop and feed during their larval stage on or inside other insects, which die at the end of the process (Godfray 1994). Among these parasitoids, hymenopterans comprise the order with the highest number of species, and it is estimated that given this specialization, this order might be between 2.5 and 3.5 times larger than coleopterans, which is the current order with the highest number of described species within the animal kingdom (Forbes et al. 2018).

In the particular case of parasitoid insects, the use of Malaise traps (Townes 1962) has been one of the most recommended and used methods (Sheikh et al. 2016) in monitoring programs, biodiversity inventories (e.g. Gauld 1991; Longino 1994; Mazón and Bordera 2008; van Achterberg 2009) and to obtain large quantities of parasitoid Hymenoptera (Sääksjärvi et al. 2004, 2006; Fraser et al. 2007; van Achterberg 2009; Lamarre et al. 2012). The Malaise trap is a passive capture system, which works by intercepting insects in flight, becoming especially adequate for capturing hymenopterans, which present positive phototropism; therefore, they fly upwards in search of light when in contact with the trap. For this reason, it is important that the collecting pot be placed towards the maximum illumination; the trap's shape leads the insect towards the collecting jar with alcohol (van Achterberg 2009).

There are several studies which have proven the efficacy of the Malaise trap regarding color (Townes 1972; Campbell and Hanula 2007), mesh size (Darling and Parker 1988), position, design, height (Darling and Parker 1988; Compton et al. 2000; Mederos-López et al. 2012) and sampling effort analysis (Castiglioni et al. 2017). One of the most important aspects for the Malaise trap efficiency is the location; the trap must be placed blocking a corridor, perpendicular to a vegetation barrier: installing them so that the base touches the ground (Sheikh et al. 2016), so its capture span ranges from the ground to about 1.50 m.

Several studies have demonstrated the differences of flying insects assemblies composition comparing the tree canopy and the ground level (Darling and Parker 1988; Compton et al. 2000; Vance et al. 2007; Mederos-López et al. 2012) or ground strata (Lamarre et al. 2012), but all of them using different kind of traps to compare the strata. However, it has not been analyzed whether placing the Malaise trap at ground level, as it is typically done, is the best option or suspending it a few meters over the ground, without reaching the tree canopy, could collect a different variety of parasitoids, considering that they do not only move around the tree canopy, but some also walk on the ground; others, especially the smaller size ones (< 1 mm long) use the air column to scatter at medium heights or over the canopy (Compton et al. 2000). With all these considerations, the aim of this work was to evaluate the diversity and size of

parasitoids in relation to the placement height of the Malaise trap above the ground in a low deciduous forest from the State of Yucatan, Mexico.

## Methods

### Study area and sampling

The present work was conducted in Hacienda Yabucu (20°48'37.55"N, 89°24'48.58"W) located in the municipality of Acanceh in the central part of the State of Yucatan, Mexico. The climate in the area is mainly warm, sub-humid with summer rains; it is one of the hottest zones in the Peninsula, with a mean annual temperature ranging from 28° to 30 °C, reaching their maximum in May (42 °C) and minimum in November (10 °C); with a rainfall from 600 to 700 mm per year. The dominant vegetation type is a low deciduous forest, with a high percentage of trees, which shed their leaves during the dry season; most of the trees are Fabaceae, with a tree layer no taller than 12 m (Rzedowski 2006).

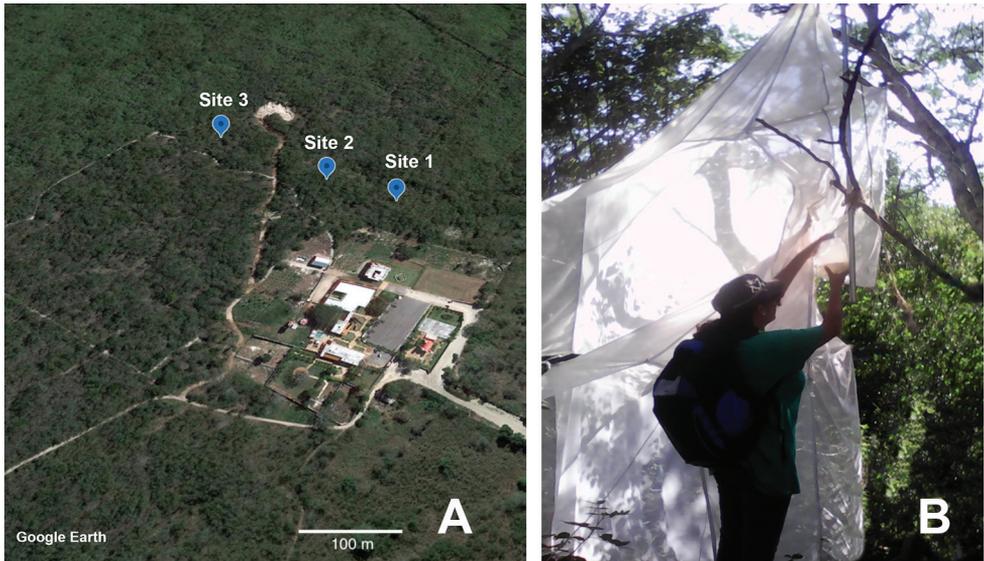
The sampling was performed from September to October 2015, because these are the months with maximum rainfall and the highest abundance peak of parasitoids in the region (González-Moreno and Bordera 2012; González-Moreno et al. 2015; González-Moreno et al. 2018). A total of six Malaise traps were placed at two different heights: three of them were placed in the conventional way, at ground level (**GMT**: Ground Malaise trap) and the others were placed immediately above the first ones, leaving no space between them, at a height of 1.5 m above ground level (**RMT**: Raised Malaise Trap) (Fig. 1). The traps functioned continuously during nine weeks, with weekly cutoffs for recollecting.

Goulet and Huber (1993), and Gibson et al. (1997) keys were used to identify parasitoids families. The collected material was deposited at the Colección Entomológica of Tecnológico Nacional de México/Campus Conkal, Yucatan.

The location map of sampling sites (Fig. 1) was downloaded from <https://www.google.es/earth/> and has been used agreeing with terms of use published in <https://www.google.com/permissions/geoguidelines/>.

### Data analysis

Family richness was described for both trap heights, considering the total of individuals per family and indicating the most abundant families in each trap position. To know how many families are expected for this method and sampling effort, the non-parametric Jackknife 1 richness estimator was calculated, which is used for small samples, with confidence intervals of 95% (Magurran 2004); using the ESTIMATES 9.1.0 software (Colwell 2014). To establish capture differences in terms of richness, a rarefaction analysis was performed, measuring the sampling effort by week and by number of individuals, adjusting it to the smallest sample.



**Figure 1.** Malaise traps position at two different heights **A** location of sampling sites in Hacienda Yabucu **B** trap placement, one placed in the conventional way, at ground level, **GMT**: Ground Malaise trap and the other placed immediately above the first ones, leaving no space between them, **RMT**: Raised Malaise Trap.

The differences in total abundance of the collected parasitoids at the two different trap heights were analyzed by a fixed effects multifactorial ANOVA, considering the time and trap positions as factors; as variances were not homogeneous, abundance data were transformed to  $\ln(x)$ , accomplishing homoscedasticity (Levene's test  $p = 0.77$ ) and residual normality (Shapiro-Wilk's  $p = 0.20$ ); this analysis was also done individually for each family, to determine if there are any differences between individuals collected at ground level and at raised level. For the comparative analysis of diversity, the non-parametric Shannon index was used, contrasted with the bootstrap method with a confidence interval of 95%.

To determine the size of the specimens collected in each trap, the forewing length (**FW**) of each specimen was measured as an indicator of body size, since both parameters are roughly positively correlated (Grimaldi and Engel 2005). To do this, the software IMAGEJ 1.45 was used, incorporating a millimetric spreadsheet as background and taking photos of each individual (Fig. 2). The size differences were analyzed by a fixed effects multifactorial ANOVA.

## Results

### Family richness

A total of 4083 specimens belonging to 31 families of parasitoid hymenopterans were collected, being Bethyliidae, Braconidae and Ichneumonidae the most abundant fami-



**Figure 2.** Measurement of FW: fore wing, using millimeter sheet.

lies, representing altogether 40% of the whole sample (Table 1). The Jackknife 1 richness estimator predicted 34 families, using six Malaise traps for nine weeks; so, we collected 93% of the expected families for this method in the low deciduous forest of Yabucu (Table 2).

In terms of family richness, the rarefaction analysis by individuals, with the lower richness estimated at 1881 individuals, was slightly superior at **RMT** with 28 families (SE = 0.12), than below, **GMT** with 26 families (SE = 0.57). This means that if both samples had equal size, raised level had the highest richness.

### Parasitoid abundance

Regarding the trap position, **GMT** collected 2202 individuals belonging to 28 families, the most abundant being Bethylidae, Ichneumonidae and Diapriidae, accounting for 54% of the total sample. The families Signiphoridae and Dryinidae were unique to this trap height (Table 1). **RMT** caught 1881 individuals belonging to 29 families, the most abundant being Braconidae, Bethylidae and Eucilidae, representing 46% of the sample. The families Ceraphronidae, Encyrtidae and Trichogrammatidae were unique to this trap height (Table 1).

There were significant differences in the number of individuals caught at different heights (Table 3). Also, when the differences of each family by separate were analyzed, there were significant differences for Bethylidae, Diapriidae and Ichneumonidae (Tables 4–6); these three families had more individuals caught at ground level than in raised traps (Table 1). This pattern remained constant during all weeks of sampling, in other words, there was no interaction between the factors trap position and time.

**Table 1.** Individuals' number collected in two Malaise trap height: **GMT, RMT** and ANOVA **F** and **p** value. Values of probabilities were significant at 0.05\*. Families without statistics values had not enough data for the analysis.

	Individuals in GMT	Individuals in RMT	Total individuals	F	p
Bethylidae	422	216	638	6.64	0.01*
Braconidae	204	365	569	1.63	0.24
Ichneumonidae	280	121	401	6.37	0.01*
Diapriidae	249	58	307	16.60	0.00*
Eucoilidae	81	200	281	2.35	0.16
Sclerogibbidae	128	148	276	0.18	0.68
Chalcididae	145	108	253	2.24	0.14
Scelionidae	125	119	244	0.07	0.80
Platygastridae	83	113	196	0.48	0.50
Eupelmidae	101	72	173	1.54	0.23
Eucharitidae	77	44	121	2.66	0.14
Evaniidae	44	53	97	0.56	0.47
Mymaridae	19	66	85	4.29	0.07
Aphelinidae	38	28	66	2.58	0.127
Eurytomidae	25	36	61	1.22	0.29
Perilampidae	37	21	58	3.45	0.10
Chrysididae	35	17	52	2.25	0.17
Sphécidae	24	22	46	0.04	0.86
Elasmidae	28	5	33	1.57	0.24
Figitidae	17	13	30	0.44	0.52
Torymidae	14	11	25	0.07	0.79
Eulophidae	8	14	22	3.24	1.09
Pteromalidae	11	9	20	2.98	0.09
Ceraphronidae	0	6	6	–	–
Gasteruptiidae	1	5	6	–	–
Rhopalosomatidae	1	5	6	–	–
Encyrtidae	0	4	4	–	–
Stephanidae	2	1	3	–	–
Dryinidae	2	0	2	–	–
Trichogrammatidae	0	1	1	–	–
Signiphoridae	1	0	1	–	–
<b>TOTAL</b>	<b>2202</b>	<b>1881</b>	<b>4083</b>		

**Table 2.** Species richness expected according Jackknife 1 estimator and percentage of specimens collected.

Site / Malaise trap height	Total of families observed	Total of families expected	% families collected
1 GMT	24.33	28.52	85.30
1 RMT	27.53	31.05	88.66
2 GMT	28.95	31.83	90.95
2 RMT	29.87	32	91.37
3 GMT	30.5	33.15	92.00
3 RMT	31	33.5	92.53

### Diversity and size of parasitoids

In terms of diversity, there were no differences between families collected at ground level (**GMT**  $H'$ : 2.68) and raised traps (**RMT**  $H'$ : 2.71) ( $p = 0.08$ ).

**Table 3.** Analysis of variance for the number of individuals of the Hymenoptera parasitoids for Malaise trap height: GMT, RMT and sampling week. Values of probabilities were significant at 0.05\*.

Main effects	Sum of squares	DF	F	p
Sampling Week	6.23	8	0.59	0.78
Malaise Height	15.61	1	11.73	0.0015*
Interaction	3.67	8	0.35	0.94
Residuals	50.7	38		
Total	96.2			

**Table 4.** Analysis of variance for the number of individuals of Bethyliidae for Malaise trap height: GMT, RMT and sampling week. Values of probabilities were significant at .05\*.

Main effects	Sum of squares	DF	F	p
Sampling week	314.50	8	0.36	0.93
Malaise Height	718.6	1	6.64	0.01*
Interaction	231.82	8	0.27	0.97
Residuals	3895.33	36		
Total	5160.31	53		

**Table 5.** Analysis of variance for the number of individuals of Diapriidae for Malaise trap height: GMT, RMT and sampling week. Values of probabilities were significant at 0.05\*.

Main effects	Sum of squares	DF	F	p
Sampling week	128.48	8	0.39	0.92
Malaise Height	675.57	1	16.60	0.00*
Interaction	98.93	8	0.30	0.96
Residuals	1464.67	36		
Total	2367.65	53		

**Table 6.** Analysis of variance for the number of individuals of Ichneumonidae for Malaise trap height: GMT, RMT and sampling week. Values of probabilities were significant at 0.05\*.

Main effects	Sum of squares	DF	F	p
Sampling week	487.37	8	0.83	0.58
Malaise Height	468.167	1	6.37	0.016*
Interaction	148.333	8	0.25	0.98
Residuals	2647.33	36		
Total	3751.2	53		

**Table 7.** Differences in individual wing length of five parasitoid families collected in traps set at different heights: GMT, RMT.

Parasitoid family	GMT	RMT	T (p)
	Mean mm (SE)	Mean mm (SE)	
Bethyliidae	2.31(.028)	1.96(0.038)	7.22(<0.05)
Ichneumonidae	4.34(.10)	5.47(0.15)	-6.35(<0.005)
Sphécidae	3.85(.18)	2.85(0.19)	3.83(<0.0005)
Sclerogibbidae	1.7(.03)	1.5(0.03)	4.9(<0.0005)
Evaniidae	2.9(.09)	2.26(0.08)	5.23(<0.0001)

Only in five of the total 31 collected families, there were differences in relation to size. The largest Bethyridae, Sphecidae, Sclerogibbidae and Evaniidae were collected at **GMT**; on the other hand, the larger Ichneumonidae were collected in **RMT** (Table 7).

## Discussion

The total of collected families comprise 97% of 32 families recorded for Yucatan Peninsula (Delfín-González and Chay-Hernández 2010) and 70% of those recorded in the Neotropic (Fernández and Sharkey 2006), resulting in an optimum sampling effort since the family richness observed (31 families) is 91% of the estimated richness (34 families). Therefore, the representativeness of the results is sufficient to make comparisons at higher taxa level, in agreement with the results by Mazon (2016) on diversity of parasitoid subfamilies. In the current study, taking into account the minimum sampling effort, the results allow to state that keeping a Malaise trap for two months provides 85% of the parasitoids' representativeness in the site at family level. However, it is important to consider the time of the year when the sampling is done, as it must coincide with the seasonal maximum populations. In temperate weathers, they follow a bimodal pattern with maxima in the Spring and Fall, or unimodal, with one population peak in Spring-Summer, related to the yearly balmy temperatures (Gaasch et al. 1998; Rodríguez-Berrío et al. 2010; Mazon et al. 2011). At the tropics, the highest abundances follow a unimodal pattern around the rainy season (Gauld 1991). Particularly for the area of study, previous works have proven that the months from August to October have the highest parasitoid abundance and diversity (González-Moreno and Bordera 2012; González-Moreno et al. 2015; González-Moreno et al. 2018).

There are several factors that affect the insect diversity among the different vertical strata of a forest; for example, time, microclimate, light intensity, movement capacity (scattering), interspecific competition, natural enemies, quality of food resources and foliage (Basset 1992). In the present study, differences in family richness and abundance were observed, probably because hymenopteran families have different searching patterns; some studies have reported that very small size parasitoids such as many Chalcidoidea, fly frequently by the vegetation canopy, taking advantage of the air column for their dispersion, whereas other families such as Mymaridae are restricted to the lower levels in the forest (Compton et al. 2000). Also, insect herbivores are more abundant and speciose in the upper canopy than in the understory (Basset et al. 2001), so it is more likely to find different families of parasitoids looking for its herbivorous insect hosts.

Two of the most abundant families were Braconidae and Ichneumonidae, which are considered hyper-diverse groups (Fernández and Sharkey 2006); furthermore, they have been recorded as the two largest families of Hymenoptera in the Yucatan Peninsula (Delfín-González and Chay-Hernández 2010) and the rest of the world (Quicke 2015), with more than 46,500 valid described species (Yu et al. 2015). In the case of Bethyridae, its abundance can be explained by the fact that they are gregarious

parasitoids (Fernández and Sharkey 2006). This means it is probable that if traps are placed where the hosts are located, a high number of individuals from this family can be caught.

Regarding exclusive families at each height level, these results should be taken with caution due to the extremely low abundances, which may not represent a preference for a given height. However, some studies have recorded preferences of Trichogrammatidae at 8 m high and Encyrtidae at 12 m (e.g., Mederos-López et al. 2012); others have recorded Encyrtidae as one of the most abundant parasitoid family at “high levels”, finding higher abundances at 18, 27 and 36 m above the ground (Compton et al. 2000). In this study, Encyrtidae, Trichogrammatidae and Ceraphronidae, fell exclusively in the traps placed at 1.5 m above the ground, which could be related to these these preferences. On another hand, the families Dryinidae and Signiphoridae fell only in traps at ground level.

The results from the analysis of families separately, demonstrated that for Bethyliidae, Diapriidae and Ichneumonidae, trap height is important to collect a better representation in terms of abundance. Bethyliidae attacks mainly Coleoptera larvae, which dwell on the ground (Vargas-Roja and Terayama 2006), so it is more likely that bethylid wasps fly near the ground. Diapriidae attacks mainly immature stages of Diptera; adults are found in humid habitats, in the shade and on the ground or near water (Masner 2006), so it is also highly probable to catch them near the ground. Lastly, for the Ichneumonidae, the difference in abundances could be also due to the host-searching strategy closer to the ground (Giraldo-Vanegas and García 1994; Kasparyan and Ruiz-Cancino 2008).

In relation to size, results suggest than this factor can also have an influence in the flight height of several families. The largest Bethyliidae, Sphecidae, Sclerogibbidae and Evaniidae were collected at **GMT**; while the larger size of Ichneumonidae was collected at **RMT**. However, the explanation of this behavior is not easy at the family level, since these groups have a large range of sizes, especially Sphecidae and Evaniidae, and particularly Ichneumonidae. A possible explanation could perhaps be found from a more detailed study of these families at the genus or species level. In this way, the biology of these species itself could explain better the differences found.

## Conclusion

In conclusion, there were no differences in the diversity of parasitoid families collected at different heights from the ground; therefore, placing a Malaise trap at ground level is an adequate method for collecting parasitoids. However, a trend to more family richness could be observed in raised traps than in those at ground level. The trap position on the ground has influence on the abundance of collected Bethyliidae, Diapriidae and Ichneumonidae, and also on the body size of Bethyliidae, Sphecidae, Sclerogibbidae, Evaniidae and Ichneumonidae. It would be convenient to conduct these studies in other types of habitats to verify if this trend persists.

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# Synonymy of *Idiococcobius* Hayat with *Coccobius* Ratzeburg (Hymenoptera, Aphelinidae): evidenced by a new species from Malaysian Borneo

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## Abstract

*Idiococcobius* Hayat, **syn. nov.**, is synonymised with *Coccobius* Ratzeburg, based on morphological and molecular data from a new species of *Coccobius* from Malaysian Borneo. The new species is sufficiently similar morphologically to the type species of *Idiococcobius* to place it unambiguously within that genus, but molecular data from the new species, and a reassessment of the morphology of *Idiococcobius*, indicate synonymy of the two genera. *Idiococcobius encarsoides* Hayat is therefore transferred to *Coccobius*; resulting in the new combination: *Coccobius encarsoides* (Hayat), **comb. nov.** *Coccobius islandicus* Geng & Polaszek, **sp. nov.** is described from morphology and DNA sequence data.

## Keywords

chalcid wasps, Chalcidoidea, parasitoids, India, Malaysia, Sabah, taxonomy

## Introduction

The genus *Idiococcobius* Hayat, 2010 was described for an unusual species of aphelinid considered to be “extremely close to *Coccobius*...” (Hayat and Khan 2010), but to have affinity with some *Encarsia* species, hence the species name *I. encarsoides* Hayat, 2010.

The morphological characters that purport to establish *Idiococcobius* as a valid genus also occur in a recently discovered new species from Malaysian Borneo. Molecular analysis of the latter species places it firmly within *Coccobius*. The affinities (and differences) between *Coccobius encarsoides* (Hayat) and *Coccobius islandicus* Geng & Polaszek, sp. nov., are detailed and discussed below.

## Materials and methods

### Morphological study

The single specimen of the new *Coccobius* species was collected in Sabah, Malaysian Borneo using a yellow pan trap. After “non-destructive” DNA extraction (see below), the specimen was dissected and mounted dorsally in Canada balsam on a slide following the method of Noyes (1982) with modifications for dealing with ProteinaseK processed specimens (AP, unpublished). Morphological terminology follows Hayat and Khan (2010).

Photographs were taken with a digital CCD camera attached to an Olympus BX63 compound microscope equipped with differential interference contrast. All measurements were taken from slide-mounted specimens. The holotype is deposited in the Natural History Museum, London, UK

The following abbreviation is used:

**NHMUK**      Natural History Museum, SW7 5BD, London, UK

### DNA sequencing

Genomic DNA extraction was undertaken using the protocol in Polaszek et al. (2013), which leaves the sclerotized parts of the specimen intact. The specimen was then mounted in Canada balsam on a microscope slide (see above). Primer sequences are given in Table 1. A total of 25 µl per reaction with 2.5 µl 10× NH<sub>4</sub> Reaction Buffer, 1.5 µl 50mM MgCl<sub>2</sub> Solution, 0.5 µl dNTP's, 1 µl of each primer, 0.5 µl BIOTAQ DNA Polymerase, and 6 µl DNA template. PCR amplifications included a pre-cycle denaturation step for 5 min at 94 °C and a final post-cycle extension step at 72 °C for 5 min (Table 1).

DNA was sequenced at the Natural History Museum Life Sciences DNA Sequencing Facility (London) using the same primers used for the PCR. Forward and reverse sequences were assembled and corrected using Sequencher version 4.8. The 28S sequence of *Coccobius islandicus* Geng & Polaszek, sp.nov., has been deposited in GenBank under accession no MT350291. The resulting COI sequence was short, and not useful for comparative analysis. However, a 28S D2 sequence of 744bp was obtained and sent (including the trace file) to the 3<sup>rd</sup> author (JM) for comparison and analysis with his substantial database of 147 *Coccobius* DNA sequences.

**Table 1.** Primer sequences and cycling conditions.

Primer sequence		Cycling conditions			
		Denaturation	Annealing	Extension	Cycles
COI (Hajibabaei et al. 2006)					
MLepF1	5' GCTTTCCCACGAATAAATAATA3'	94 °C	50 °C	72 °C	40
LepR1	5' TAAACTTCTGGATGTCCAAAAAATCA 3'	(30 sec)	(30 sec)	(1 min)	
28S-D2 (Park and O'Foighil 2000)					
D23f	5' GAGAGTTCAAGAGTACGTG 3'	94 °C	50°C	72 °C	40
28Sb	5' TCGGAAGGAACCAGCTACTA 3'	(30 sec)	(30 sec)	(1 min)	

The 28S sequence from *C. islandicus* was incorporated into a larger molecular dataset (JM in-prep; data not shown) that included an encyrtid outgroup (four terminals, from the genera *Anagyrsus*, *Encyrtus*, *Metaphycus*, and *Oobius*), non-*Coccobius* coccophagine aphelinids (20 terminals, from the genera *Bardylis*, *Coccophagoides*, *Coccophagus*, *Dirphys*, *Encarsia*, *Euxanthellus*, and *Pteroptrix*), and 147 *Coccobius* terminals representing specimens from all biogeographic regions where *Coccobius* are known. Ribosomal DNA sequences were aligned using the E-INS-I algorithm in Mafft v6 (Kato et al. 2009) with default settings. Ribosomal and mitochondrial genes were then concatenated using SequenceMatrix v.100.0 (Vaidya et al. 2010) for a final alignment length of 3153 base pairs. Maximum likelihood (ML) analysis of the concatenated alignment was conducted using RAxML v8.2.12 (Stamatakis 2014) under a GTR+ $\Gamma$  substitution model as implemented through the CIPRES Web Portal (Miller et al. 2010) accessed at <http://www.phylo.org/>. The data were analyzed with 1000 rapid bootstraps using five gene partitions: *18S*, *28S-D2*, *28SD3-5*, *COI* codon positions 1 and 2, and *COI* codon position 3. Only the 28S sequence from *C. islandicus* aligned with the existing dataset, so only its 28S sequence is included in the analysis. The resulting best tree (highest likelihood) was visualized using FigTree v1.4.3 (Rambaut 2009).

## Results

### Morphology

*C. islandicus* Geng & Polaszek, sp. nov., shares a number of apparent synapomorphies with *C. encarsoides* (Hayat) as follows: pronotum medially membranous; mid lobe of mesoscutum with a reduced number of setae arranged bilaterally; fore wing narrow with very long marginal fringe (more than 2/3 the maximum wing width); submarginal vein with a single seta. These characters, not previously encountered in described *Coccobius* species, clearly indicate that the two species are closely related.

The morphological differences between the two species, tabulated below, strongly support recognition of *C. islandicus* as a new species.

**Table 2.** Differences between *C. islandicus* (Geng & Polaszek) and *C. encarsoides* (Hayat).

	<i>C. islandicus</i> (Malaysia)	<i>C. encarsoides</i> (India)
colour of body	pronotum brown, lateral metasoma with brown patches; remainder pale.	body largely pale yellow, appearing translucent white after cleaning and mounting except pronotum in anterior half pale brown
colour of antenna	F1 and club brown; remainder white.	entirely white
colour of legs	fore and mid tibiae pale brown, hind femora and tibiae brown; remainder white.	white
ovipositor/mid tibia	1.21	1.49
third valvula/ovipositor	0.27	0.23
mid tibial spur/ mid basitarsus	1.29	1.12
fore wing length/ width	3.22	4.4
fore wing marginal fringe/width	0.68	slightly shorter
setae on mid lobe of mesoscutum	8	12
setae on TII of metasoma	1+1	2+2
sensilla on stigmal vein	in a close group of 4	in 2 separated groups of 2

## Molecular analysis

The analysis of the 28S D2 sequence of *C. islandicus* together with 147 *Coccobius* sequences representing at least 125 species unequivocally demonstrates that this species, and by extrapolation therefore *Idiococcobius*, are nested deeply within *Coccobius* and do not represent a distinct genus. *Idiococcobius* Hayat is therefore here synonymized with *Coccobius* Ratzeburg, syn. nov. *Idiococcobius encarsoides* Hayat is here transferred to *Coccobius encarsoides* (Hayat), comb. nov. *C. islandicus* clusters with other Australian / SE Asian species. Its sister species (with low bootstrap support) is from Singapore.

## Taxonomy

### *Coccobius islandicus* Geng & Polaszek, sp. nov.

<http://zoobank.org/EE1EE627-07A5-4DFD-B7C1-CB70AD20A853>

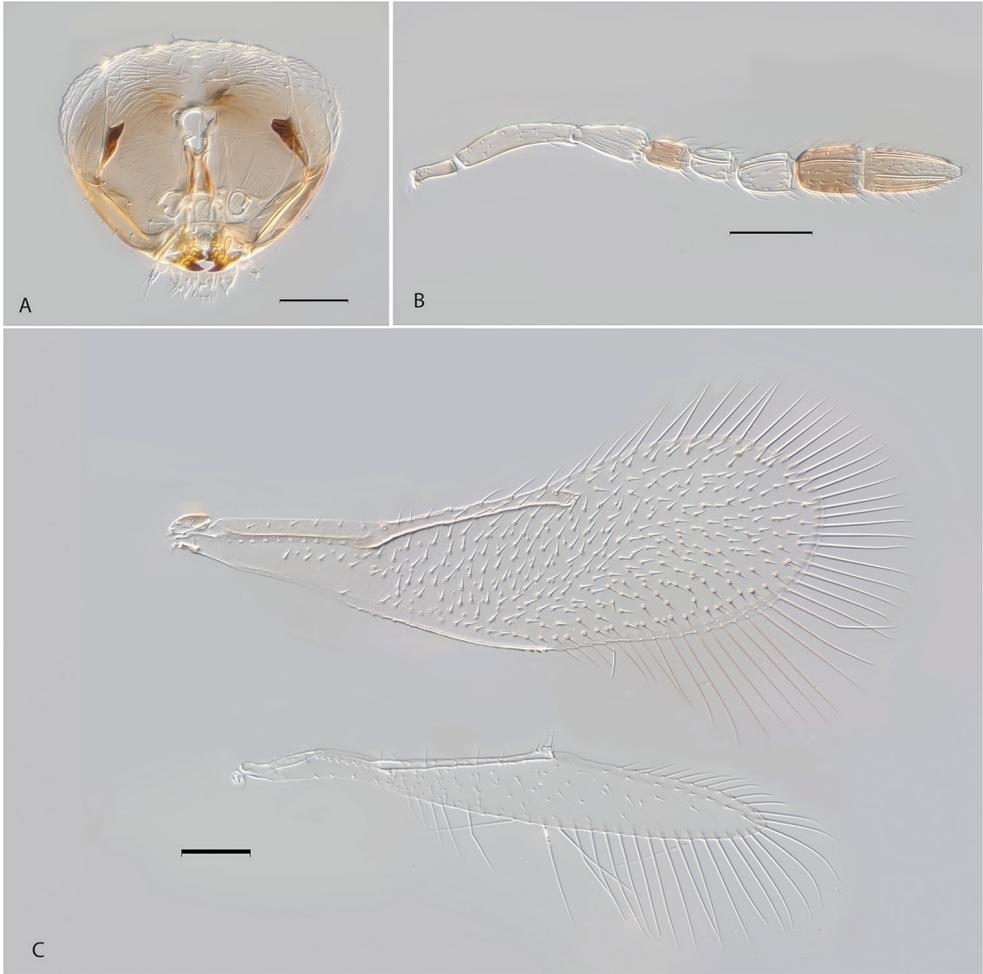
Figs 1 A–C, 2 A, B

**Material examined.** Holotype ♀ (on slide): 21–25 Aug 2016, MALAYSIA: Sabah (N. Borneo), Keningau, Jungle Girl Camp, 5°26'55.7"N, 116°27'08.6"E, rainforest. H. Geng; NHMUK; specimen ID: GH025-92

**Diagnosis.** Antennal F1 and clava brown (Fig. 1B). Pronotum medially membranous; mid lobe of mesoscutum with 8 setae (Fig. 2A); maximum length of marginal fringe of fore wing 0.68× wing width.

**Description (female holotype).** Length 0.60 mm.

**Colour.** Head brown, frons paler. Mandibles very dark, especially toward apices (Fig. 1A). Antenna pale, F1 and clava brown (Fig. 1B). Pronotum and anterior margin

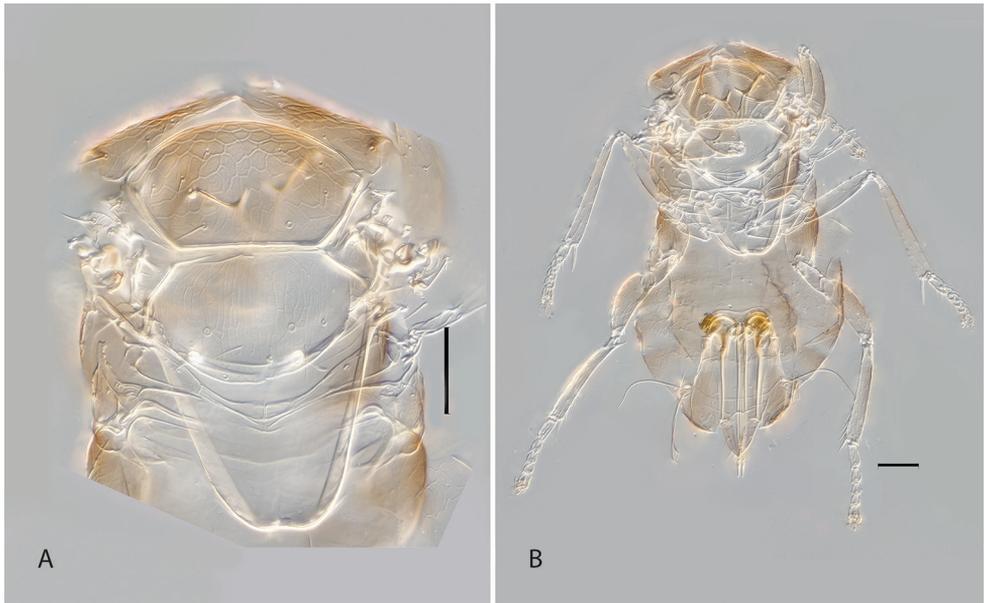


**Figure 1.** *Coccobius islandicus* Geng & Polaszek, sp. nov. **A** head **B** antenna **C** wings. Scale bars: 50 µm.

of mesoscutal mid lobe brown, remainder of body largely pale, but brown laterally. Fore and mid tibiae pale brown, hind femora and tibiae brown (Fig. 2B). Wings hyaline except tegula pale brown (Fig. 1C).

**Morphology.** Head 1.5 times as broad as frontoververtex width (Fig. 1A), frontoververtex width less than length of clava (0.74). Upper margins of antennal toruli slightly below lower eye margins. Eye setose. Antennal radicle long,  $0.37\times$  scape length. Pedicel just over  $0.5\times$  scape length,  $1.56\times$  length F1. F1 as long as F2,  $0.78\times$  F3. Clava slightly longer than funicle. Antennomeres with the following numbers of sensilla: F1:0; F2:1; F3:2; C1:3; C2:5.

Mid lobe of mesoscutum with 8 setae arranged in approximate bilateral symmetry. Sculpture of mesoscutal mid lobe consisting of large irregular reticulations, that of scutellum with longitudinal elements. Scutellum with 2+2 setae, the front pair placed



**Figure 2.** *Coccobius islandicus* Geng & Polaszek, sp. nov. **A** mesosoma **B** mesosoma- and metasoma. Scale bars: 50  $\mu$ m.

slightly behind the level of the scutellar sensilla (Fig. 2A). Wings elongate. Fore wing 3.22 $\times$  as long as wide, marginal fringe 0.68 $\times$  as long as maximum wing width, sub-marginal vein with 1 seta, stigmal vein sensilla in a cluster of 4 (Fig. 1C). Hind wing 7 $\times$  as long as wide, marginal fringe 1.6 $\times$  as long as maximum wing width. Mid tibial spur 1.29 $\times$  mid basitarsus.

Metasomal setation: T1: 0+0; T2:1+1; T3-T5: 2+2; T6:4; T7:6. Ovipositor length 1.21 $\times$  mid tibia; 3<sup>rd</sup> valvula 0.27 $\times$  ovipositor (Fig. 2B).

**Male.** Unknown.

**Host.** Unknown.

**Distribution.** Sabah, Malaysian Borneo.

**Etymology.** "island"-(*icus*) is the latinised form of the English word "Iceland", in this case referring to a British food retailer that has banned the use of palm oil in its products. The species name does not imply any endorsement by the authors of this retailer's products.

## Discussion

Hayat (in Hayat and Khan 2010) described *Idiococcobius* as a genus he considered to be close to, but distinct from, *Coccobius*, based on a combination of characters that had not previously been encountered in any *Coccobius* species known to him at that time. The medially membranous pronotum, reduced and bilaterally symmetrical meso-scutal setae, and narrow fore wing with long marginal fringe, all suggested possible affinity with *Encarsia*, a genus not particularly close to *Coccobius*. These characters

clearly merited the description of a new genus, with the *Encarsia* similarities conveyed in the species name “*encarsoides*”. These kinds of reduction characters appear to be common not only across the Aphelinidae, but among many chalcids and beyond. Setal reduction and wing narrowing (as seen here), reduction in tarsal segment number and antennomeres are all processes known to have occurred independently in multiple lineages, and have confounded traditional morphology-based taxonomy for so long. The development of relatively comprehensive molecular databases, even those (as herein) relying on a single gene, are extremely valuable for the unequivocal elucidation of an organism’s true relationships. While not always perfect, when sample size is adequate and gene variability sufficient, their predictive power is considerable. Studies such as that described above are able both to contribute towards more stable classification as well as reveal widespread morphological convergence, especially in organisms with similar biology.

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# Biological attributes of diapausing and non-diapausing *Doryctobracon areolatus* (Hymenoptera, Braconidae), a parasitoid of *Anastrepha* spp. (Diptera, Tephritidae) fruit flies

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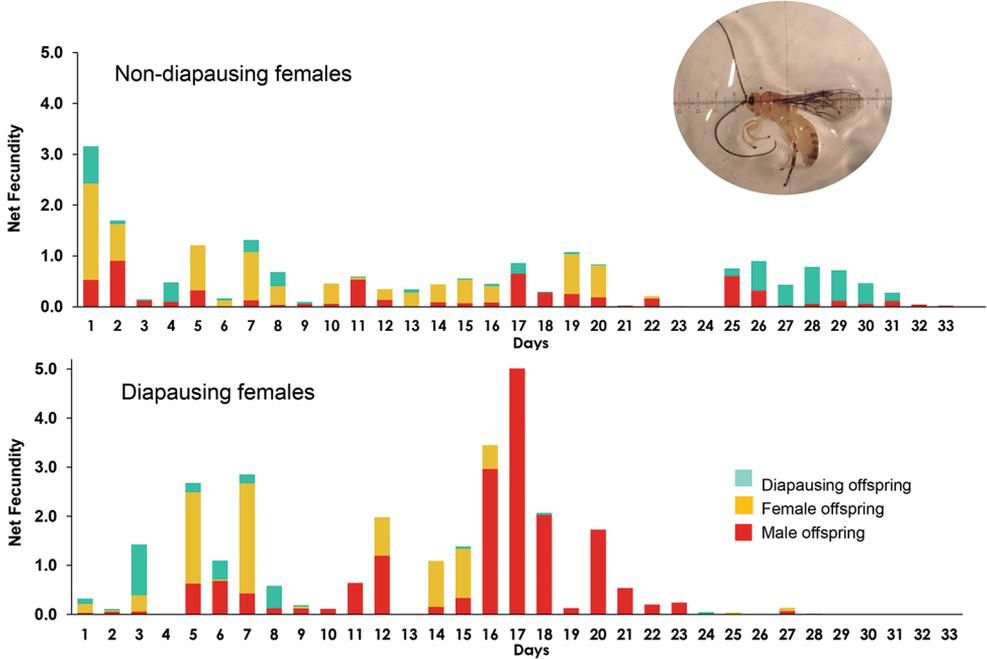
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## Abstract

*Doryctobracon areolatus* (Szépligeti), a solitary endoparasitoid native to the Neotropics, attacks eggs and early instar larvae of *Anastrepha* fruit flies, and can enter diapause under tropical and subtropical conditions. We aimed to test if biological attributes, such as size, flight ability, starvation resistance, longevity and fecundity of diapausing individuals differ from those of non-diapausing ones. Parasitoids were obtained from a laboratory colony reared on *Anastrepha ludens* (Loew) larvae. Parasitized host puparia were sorted in two cohorts according to their diapause condition. Developmental time from egg to adult ranged from 18 to 31 days in non-diapausing parasitoids, and 70 to 278 days for diapausing individuals. Pupal weight and adult measurements were higher in non-diapausing than in diapausing parasitoids. There were no differences in adult longevity, starvation resistance, and emergence between diapausing and non-diapausing wasps. Flight ability and fecundity rates were greater in the non-diapausing than in the diapause cohort. The proportion of female offspring was greater in the non-diapausing cohort (42.5%), whereas in the diapausing cohort the male offspring proportion was greater (62.4%). Both cohorts produced diapause offspring, but the non-diapausing cohort produced more (26.6%) than the diapausing one (9.1%). Maternal age had a significant effect on the proportion of diapause offspring: in 26 to 34 days old non-diapausing females, 78.9% of their offspring entered into diapause. These results confirmed that diapause affects the

biological attributes of *D. areolatus*. The observed differences contribute to better understand the diapause influence on the colonization and rearing process of this species and its use as biocontrol agent.

## Graphical abstract



## Keywords

Braconidae, fecundity, flight ability, size, Summer diapause, survival, Tephritidae

## Introduction

Tephritid fruit fly parasitoids are grouped in five families of Hymenoptera: Braconidae, Diapriidae, Eulophidae, Figitidae, and Ichneumonidae (Ovruski et al. 2000). This represents a wide group that exploits different immature stages of their hosts (eggs, larvae, and pupae) with important variations in their biology and behaviour (Godfray 1994). Since these parasitoids are holometabolous, each species has a different rate of development, which also largely depends on the host (stage, instar, condition). Some species may diapause (Aluja et al. 1998; Carvalho 2005; Ovruski et al. 2016).

Diapause is defined as a type of dormancy in which metabolic and developmental arrest occur in the life cycles of many invertebrates (Mansingh 1971). This allows them to survive in periods of environmental adversity, exploit fluctuating seasonal resources, and synchronize their reproduction (Košťál and Denlinger 2011). Diapause is considered an adaptive strategy in insects that live in temperate regions for winter survival

(Denlinger 2002; Košťál and Denlinger 2011). However, it has also been observed in organisms that live in tropical regions (Denlinger 2002). Several species of tephritid fruit fly parasitoids native to the Neotropics have been reported to diapause: *Doryctobracon areolatus* (Szépligeti), *Doryctobracon brasiliensis* (Szépligeti), *Utetes anastrephae* (Viereck), *Opius bellus* (Gahan) as well the introduced species *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae), *Aganaspis pelleranoi* (Brèthes) and *Odontosema anastrephae* (Borgmeier) (Hymenoptera: Figitidae) (Aluja et al. 1998; Carvalho 2005; Ovruski et al. 2016). It has been reported that *D. areolatus* is the species that diapauses with greater frequency and with the longest duration: up to 11 months in fruits of *Spondias mombin* L. (Anacardiaceae) in Mexico (Aluja et al. 1998), and 414 days in fruits of *Eugenia uniflora* L. (Myrtaceae) in Brazil (Carvalho 2005).

The genus *Doryctobracon* Enderlein, 1920, is endemic to the Americas (Ovruski 2003). *Doryctobracon areolatus* is a solitary endoparasitic koinobiont wasp that is widely distributed from Mexico to Argentina and is present in Florida (Wharton and Marsh 1978; Murillo et al. 2015). This species can oviposit in eggs and larvae of 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> instars of *A. obliqua* (Macquart) (Murillo et al. 2015). Besides, it has been reported parasitizing *A. ludens* Loew, *A. striata* Schiner, *A. fraterculus* Wiedemann, *A. serpentina* Wiedemann, *A. bahiensis* Costa Lima, *A. crebra* Stone, *A. spatulata* Stone and *Rhagoletis pomonella* Walsh, in Mexico (Aluja et al. 2013).

*Doryctobracon areolatus* developmental time, when it does not diapause, is 27 days (Murillo et al. 2015); adults usually emerge in synchrony with their host. Under laboratory conditions and optimal diet, adult females live around 20 days (Stuhl et al. 2011; Aluja et al. 2013). This neotropical species shares an evolutionary history with *Anastrepha* fruit flies (Aluja et al. 1998; Ovruski et al. 2000; Carvalho 2005).

The study of diapause in fruit fly parasitoids is relevant not only because of its potential to infer evolutionary relationships, but also to provide insights into the use of these species as biocontrol agents. Biological characteristics of diapausing individuals such as reproductive capacity, tolerance to environmental stress or flight ability can influence their use in biological control projects (Denlinger 2002, 2008; Košťál and Denlinger 2011).

Our aim in this research was to determine if there are differences in *Doryctobracon areolatus* between biological attributes (size, flying ability, starvation resistance, longevity, fecundity, offspring sex ratio and diapause frequency) of individuals which diapause and directly developing individuals, to infer if this condition influences the fitness of this species, and the implications for its mass rearing.

## Methods

### Biological material

Parasitized host puparia and adult parasitoids came from the *D. areolatus* colony that is maintained in the Biological Control laboratory of the Moscafrut Program (SADER-SENASICA), located in Metapa, Chiapas, Mexico, which at the time of bioassays had

23 generations under laboratory rearing conditions. Second instar larvae of *A. ludens* were used as hosts. Puparia were placed in 30 ml plastic containers, covered with organza fabric to allow ventilation. They were kept on a coconut fiber substrate that was kept slightly humid with water applied by spraying until adult emergence. Laboratory conditions were  $24 \pm 1$  °C temperature, 80–90% relative humidity and a 12:12 L:D cycle.

### **Developmental time**

Two cohorts were obtained based on the type of development of the parasitoids: host puparia with parasitoids without diapause (direct development), and host puparia with evidence of parasitoids in diapause (hereafter non-diapausing and diapausing, respectively). The puparia containing larvae of diapausing parasitoids were distinguished by observing the 3<sup>rd</sup> instar parasitoid larva inside the host pupa under a stereomicroscope. We recorded the developmental time for both cohorts.

### **Pupal and adult measurements**

Fly puparia were individually weighed using an analytical scale (Ohaus, Pine Brook, NJ) and then placed in plastic containers with 24 independent cells. Each cell was conditioned with lightly moistened coconut fiber substrate, where they remained under laboratory conditions until adult emergence.

Pupal and adult measurements were made with a stereomicroscope (Carl Zeiss, Stemi 2000C) fitted with a scale in the right eyepiece. Thirty host puparia containing parasitoid larvae of each physiological condition were randomly selected and the width and length, from the end of the buccal carinae to the end of the anal pore, were measured. For adult measurements, the cells were checked daily, recording for each emerged parasitoid the date and sex. Each individual was placed in a 1.5 ml vial with an 80% alcohol solution. We measured: 1) length of the left posterior tibia, 2) length of the left wing, 3) mesosoma length, 4) metasoma length, 5) antenna length, and 6) ovipositor length (Sagarra et al. 2001).

### **Emergence and flight ability**

Samples of 100 diapausing and 100 non-diapausing pupae were placed inside a 10 cm diameter X 10 cm height PVC tube, painted black, with the inner wall of the tube covered with neutral talcum powder to prevent the outflow of non-flying parasitoids (as in FAO/IAEA/USDA 2019). These devices were placed inside a 65 × 65 × 45 cm metal frame cage covered with organza fabric (SENASICA 2012). From the beginning of adult emergence, observations were made every 12 hours. Adult parasitoids that were able to fly out of the tube were collected and their number and sex were recorded. The number of non-emerged parasitoids, parasitoids that opened

the puparium but could not get out of it, and non-flying parasitoids (those that despite having emerged completely from the puparium did not manage to leave the tube) were recorded (SENASICA 2012). The number of parasitoids that remained in diapause was also recorded. The number of diapausing parasitoids was subtracted from the initial number to determine the percent of emergence and percent of flyers. There were two replicates for direct development individuals and three replicates for diapausing individuals.

### Longevity and starvation resistance

Adults that emerged from both diapausing and non-diapausing larvae were individually placed in 10×12×16 cm plastic cages. Honey embedded in towel paper placed on a plastic lid (1.5 × 0.07 cm) was provided as food (Montoya et al. 2012). Water was supplied in 20 ml plastic containers with a cone of absorbent paper. Thirty males and 30 females of each condition were observed. Daily, the number of dead individuals, type of development and their sex were recorded.

For starvation resistance, at emergence, 30 males and 30 females for each type of development (direct development and diapause) were placed in plastic cages (10×12×16cm) without food and water. Daily, the cages were checked and the dead parasitoids were collected and recorded, noting their type of development, sex and age.

### Fecundity

Forty pairs of adults emerged from both diapausing and non-diapausing larvae were used. Each pair was placed in a 10×12×16 cm plastic cage. The individuals were provided with food (honey) and water as described above. The food was changed twice a week. To evaluate fecundity we used artificial oviposition units made of a guava fruit (*Psidium guajava* L.) (Myrtaceae) (Aluja et al. 2009, López-Arriaga et al. 2020), where the mesocarp and seeds were removed and a layer of approximately 5 mm of epicarp was left. The space inside the fruit was filled with a 2.5 cm in diameter plastic sphere and 30 *A. ludens* larvae of second instar mixed with larval food. The spheres had a hole in the center through which a piece of galvanized wire 7 cm long was passed, to hang the device inside the cage. One oviposition device was placed in each cage and they were replaced every 24 hours until the death of the female.

*Anastrepha ludens* larvae were removed from the oviposition devices and placed in containers with larval diet for seven more days. Then, the mature larvae were separated from the food with a sieve and water and placed in plastic bottles with moist coconut fiber to promote pupation. At emergence, the number and sex of the emerged adult parasitoids were recorded. In the case of non-emerged pupae, they were examined under a stereomicroscope to determine if they were in diapause, dead or were unparasitized *A. ludens* pupae. For each female we recorded the number of offspring produced per day, noting males, females and diapausing individuals.

## Statistical analysis

Differences in developmental time (mean  $\pm$  SE) were analyzed by a *t* test. Morphometric data (mean  $\pm$  SE) were analyzed using a canonical multivariate analysis of variance (MANOVA) (Fay and Shaw 2010), and multiple comparisons were made using the first two canonical variables (Friendly and Fox 2017). Flight ability (mean  $\pm$  SE) was analyzed by a *t* test. Longevity data were subjected to a survival analysis (Log-rank) (Therneau and Grambsch 2000) with interval-censored data and multiple comparisons by orthogonal contrasts with Bonferroni correction, with a level of significance of 5%. Life tables were constructed with data from the survival and fecundity tests. The survival of females was analyzed by an asymptotic Log-rank test for interval-censored data (Therneau 2015). All analyses were performed using the Software R version 3.4.2. (Venables and Ripley 2002; Fox 2005, 2017; Fox and Bouchet-Valat 2017; R Core Team 2017).

## Results

### Developmental time

A total of 5,832 host puparia with evidence of diapausing parasitoids, and 934 puparia with non diapausing parasitoids were used in the bioassays. Developmental time from egg to adult, which was from the exposure of the host (*A. ludens* second instar larvae) to adult emergence, ranged from 70 to 278 days for diapausing parasitoids and from 18 to 31 days in non-diapausing ones (Fig. 1). Table 1 shows the mean ( $\pm$  SE) parasitoid development time and mean weight ( $\pm$  SE) of parasitized host puparia that yielded female and male parasitoids in each development condition.

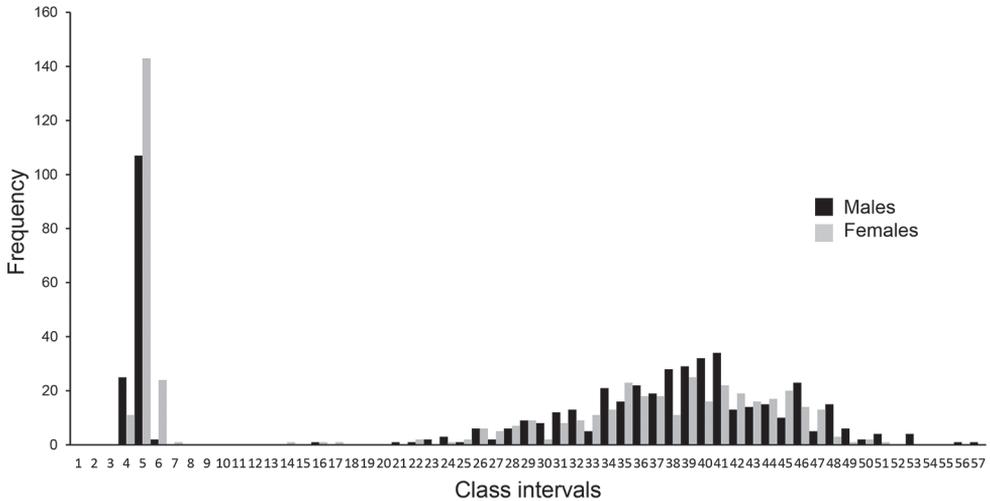
### Pupal and adult measurements

The multivariate canonical analysis, considering the length, width and weight of the host puparia containing wasps in diapause and wasps without diapause, indicated a significant interaction of sex and type of development (Manova,  $F_{3,115} = 3.86$ ,  $P < 0.01$ ). The host puparium weight of non-diapausing parasitoids was greater than that of diapausing ones. However, host puparia of diapausing female parasitoids tended to be longer and wider, but the only significant difference was in puparium length when compared with non-diapausing females. Likewise, host puparia from which female parasitoids emerged were heavier, longer and wider than the puparia containing males (Table 1, Fig. 2).

Regarding parasitoid size, statistical differences were found in the type of development ( $F_{5,74} = 7.06$ ,  $P < 0.0001$ ) and sex ( $F_{5,74} = 17.78$ ,  $P < 0.0001$ ), but there was not a significant interaction between these two factors ( $F_{5,74} = 1.07$ ,  $P > 0.05$ ). Parasitoids directly developing had longer tibia, wing, abdomen, and antenna, compared to parasitoids that diapaused (Table 1, Fig. 3A). Among females, non-diapausing individuals

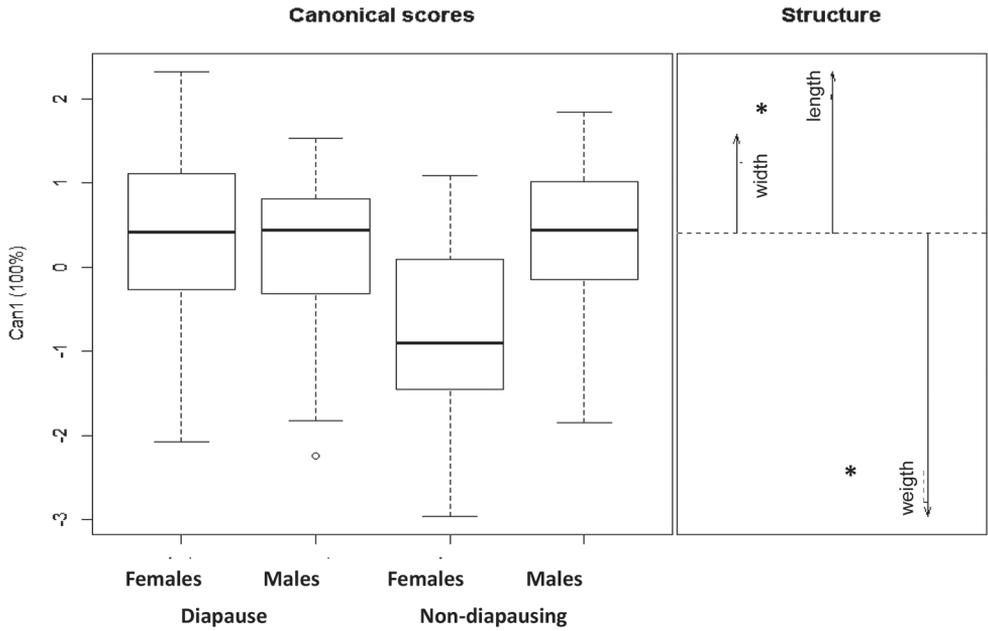
**Table 1.** Developmental time and morphological measurements of non-diapausing and diapausing *Doryctobracon areolatus* parasitoids and host puparia. SE: Standard error,  $n$  = sample size. Values followed by different letters in each row are significantly different ( $P < 0.05$ , canonical discriminant analysis).

Type of development	Non-diapausing				Diapause				
	Sex	♂	♀	♂	♀	♂	♀		
Parameter		Mean ± SE	$n$	Mean ± SE	$n$	Mean ± SE	$n$	Mean ± SE	$n$
Development time (days)		21.86 ± 0.16 c	134	23.35 ± 0.15 b	179	191.60 ± 1.62 a	384	188.85 ± 1.76 a	317
Puparia weight (mg)		13.1 ± 0.5 ab	29	15.0 ± 0.6 c	37	12.0 ± 0.4 ab	32	14.0 ± 0.5 a	26
Puparia length (mm)		6.25 ± 0.15 ab	29	6.39 ± 0.09 c	37	6.24 ± 0.06 ab	32	6.52 ± 0.08 a	26
Puparia width (mm)		2.84 ± 0.03 a	29	2.88 ± 0.04 a	37	2.81 ± 0.03 a	31	2.92 ± 0.04 a	24
<b>Adults</b>									
Tibia length (mm)		1.53 ± 0.02 a	27	1.56 ± 0.02 a	28	1.46 ± 0.03 b	13	1.52 ± 0.02 a	14
Wing length (mm)		4.88 ± 0.04 b	27	5.13 ± 0.08 a	28	4.48 ± 0.07 c	13	4.89 ± 0.04 b	14
Thorax length (mm)		2.03 ± 0.03 a	27	2.13 ± 0.04 a	28	1.99 ± 0.03 a	13	2.29 ± 0.24 a	14
Abdomen length (mm)		3.37 ± 0.07 a	27	3.30 ± 0.06 a	28	3.31 ± 0.07 a	13	3.09 ± 0.09 b	14
Antenna length (mm)		7.64 ± 0.10 a	27	7.27 ± 0.10 b	28	7.08 ± 0.10 b	13	6.64 ± 0.11 c	14
Ovipositor length (mm)		–		4.92 ± 0.07 a	28	–		4.69 ± 0.24 b	14

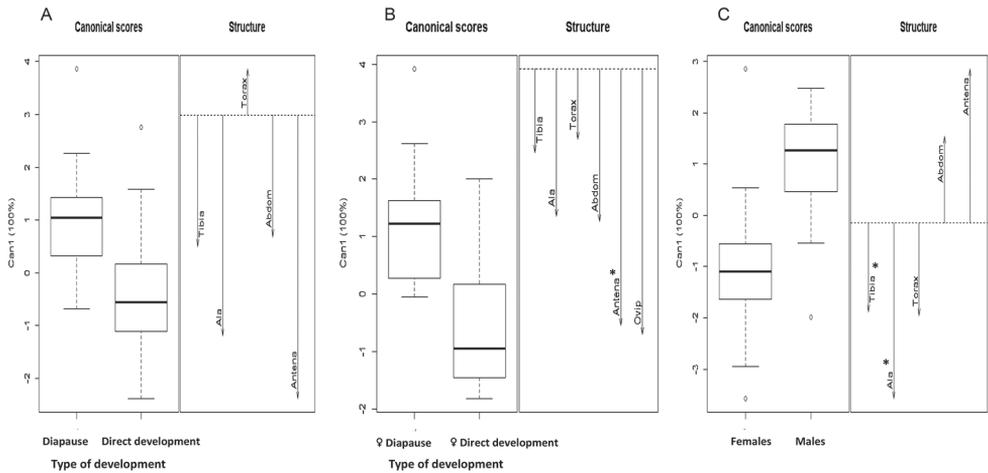


**Figure 1.** Duration of development of non-diapausing and diapausing *Doryctobracon areolatus* females and males, parasitizing *Anastrepha ludens* larvae.

were larger than diapausing ones in wing, abdomen, antenna, and ovipositor lengths (Table 1, Fig. 3B). Male parasitoids from direct development had longer tibia, wing and antenna than diapausing males. Regardless of the type of development, females were larger than males in tibia, wing, and mesosoma length (Table 1, Fig. 3C). The canonical analysis, considering all six adult parasitoid measurements, showed that non-diapausing parasitoids were significantly larger than diapausing ones.



**Figure 2.** Canonical analysis of morphological data from puparia containing non-diapausing and diapausing male and female *Doryctobracon areolatus* parasitoids. The asterisk (\*) indicates a significant difference.



**Figure 3.** Canonical analysis of adult parasitoids' morphological data from non-diapausing and diapausing *Doryctobracon areolatus*. **A** Comparison by type of development **B** comparison among females **C** comparison between sexes. The asterisk (\*) indicates a significant difference.

**Emergence and flight ability**

Adult emergence rate was higher in non-diapausing parasitoids (75.69% ± 2.96) than in diapausing parasitoids (39.86% ± 9.69). However, the difference was not statistically significant (Student t test,  $t = 2.82$   $df = 3$ ,  $P > 0.05$ ). The percentage of flying

**Table 2.** Flight ability and emergence rate of non-diapausing and diapausing *Doryctobracon areolatus* parasitoids. Emergence rate is the proportion of adults emerged from 100 host puparia. Percentage of fliers is the proportion of parasitoids capable of flying from 100 host puparia. SE = Standard Error. Values followed by different letters in each column are significantly different ( $t = 4.03$ ,  $df = 3$ ,  $P < 0.05$ ).

Cohort	Replicates	Emergence rate (%) $\pm$ SE	Fliers (%) $\pm$ SE
Non-Diapausing	2	75.69 $\pm$ 2.96 a	55.56 $\pm$ 3.54 a
Diapausing	3	39.86 $\pm$ 9.69 a	23.36 $\pm$ 5.36 b

parasitoids from direct development (55.56%  $\pm$  3.54) was significantly higher than in diapausing parasitoids (23.36%  $\pm$  5.36) ( $t = 4.03$ ,  $df = 3$ ,  $P < 0.05$ ) (Table 2).

### Longevity and starvation resistance

There were significant differences in survival between starved individuals and those provided with food (Log-rank test,  $\chi^2 = 98.46$ ,  $df = 7$ ,  $P < 0.001$ ). When food was provided, diapausing females showed the greatest mean longevity (24 days, Table 3). Longevity of diapausing females was significantly different from the longevity of diapausing males ( $Z = 3.46$ ,  $P = 0.0005$ ) but it was not different from that of non-diapausing males and females.

No significant differences were found in starvation resistance between diapausing and non-diapausing cohorts, nor between females and males (Table 3).

In the fecundity bioassays when females were provided with hosts, there was no difference in the survival of the females of both conditions ( $Z = 1.24$ ,  $P > 0.05$ , Fig. 4).

### Fecundity

Differences in the reproduction of parasitoids emerged from direct development and diapause were observed, both in the fecundity rates and in the allocation of the offspring. Fecundity was higher for the non-diapausing cohort than for the diapausing one. Non-diapausing females also produced more daughters than diapausing ones and more individuals entering into diapause (Table 4). Females of both cohorts started reproduction from the first day of adult life. In non-diapausing females, daily net fecundity was at its maximum on day 1 (Fig. 5), and their female offspring represented 42.58% of the total offspring. In diapausing females, the peak fecundity was observed on day 17 and males represented 62.54% of the total offspring. Both cohorts produced offspring that entered into diapause, 26.55% in non-diapausing females and 9.12% in diapausing females (Table 4).

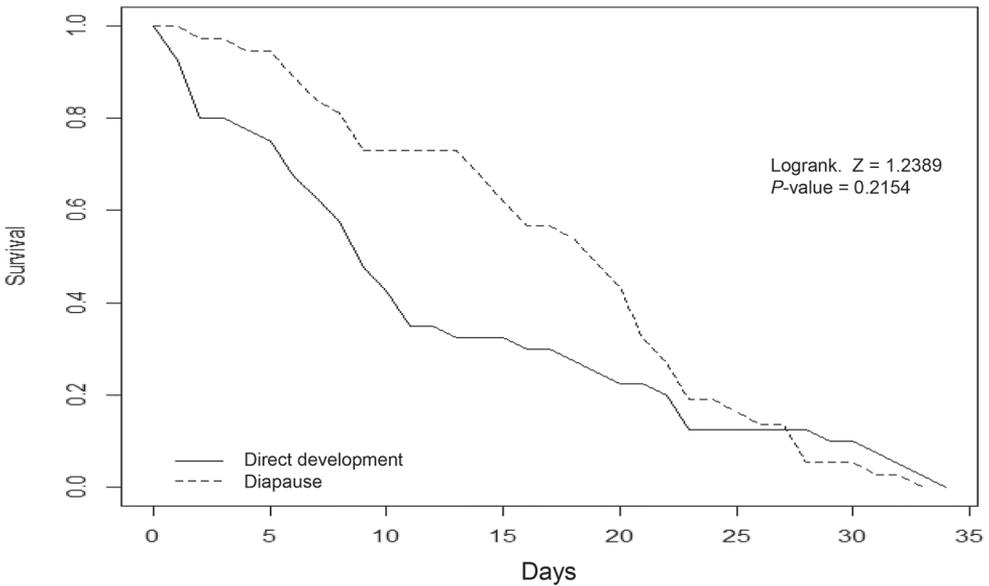
About 67% of both diapausing and non-diapausing pairs produced offspring. Of those pairs with offspring, 86.2% of the non-diapausing cohort and 100% of the diapausing cohort produced males, whereas 62% and 48%, respectively, produced females. There were no differences in the number of non-diapausing and diapausing pairs that produced daughters ( $\chi^2 = 1.07$ ,  $P > 0.05$ ). The fraction of

**Table 3.** Mean longevity ( $\pm$  SE) and starvation resistance (in days) in non-diapausing and diapausing *Doryctobracon areolatus* parasitoids.

Sex	Type of development	Longevity (days)			
		With food	n	Without food	n
♀	Non-diapausing	14.77 $\pm$ 2.27 ab	43	4.18 $\pm$ 0.33 a	55
	Diapausing	24.00 $\pm$ 3.04 a	30	5.40 $\pm$ 0.27 a	45
♂	Non-diapausing	12.09 $\pm$ 1.48 ab	47	4.40 $\pm$ 0.40 a	43
	Diapausing	10.67 $\pm$ 1.35 b	52	4.33 $\pm$ 0.24 a	52

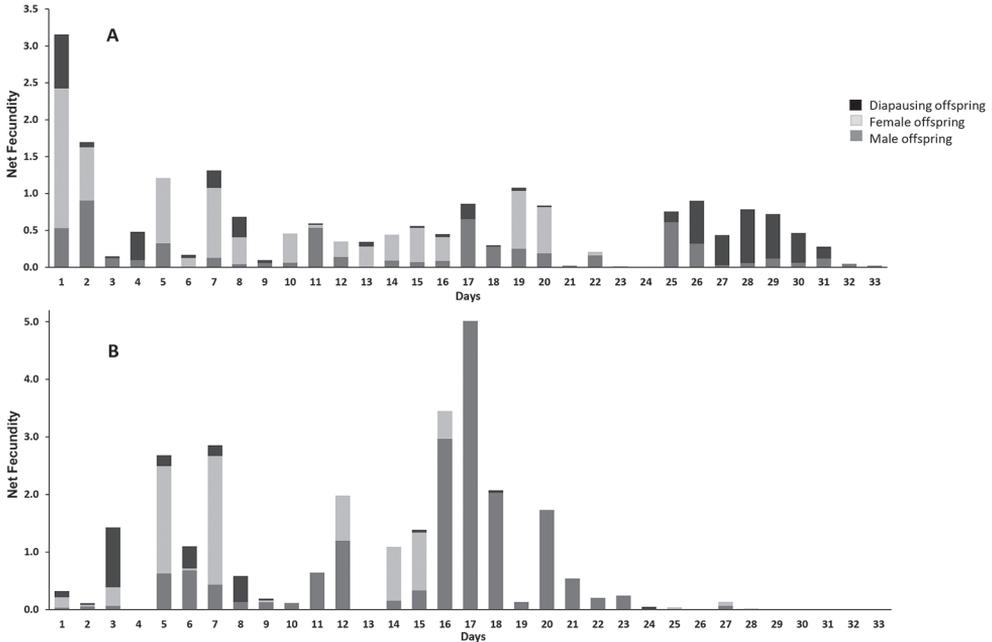
**Table 4.** Fecundity rates (female offspring per female) of non-diapausing and diapausing *Doryctobracon areolatus* parasitoids and fraction of females, males and diapausing offspring.

	Non-diapausing	Diapausing
Gross fecundity (daughters / female)	19.99	10.92
Net fecundity (daughters / female)	8.47	8.00
Male offspring (%)	30.87	62.54
Female offspring (%)	42.58	28.49
Diapausing offspring (%)	26.55	9.12



**Figure 4.** Female survival of *Doryctobracon areolatus* parasitoids in the fecundity bioassays.

pairs that produced offspring that entered into diapause was 58% and 40% for the non-diapausing, and diapausing cohorts, respectively. Maternal age in the non-diapausing cohort had an important effect on the production of offspring that entered into diapause. Over 78% of the offspring of 26 to 34 days-old females from



**Figure 5.** Net fecundity of *Doryctobracon areolatus* females from **A** non-diapausing and **B** diapausing cohorts.

the direct development condition entered into diapause, and the rest were only males (Fig. 5). Diapausing females that reached this age range did not produce offspring entering diapause.

## Discussion

Diapausing and non-diapausing *D. areolatus* individuals differed in pupal and adult size, flight ability, fecundity, and reproductive dynamics, whereas there were no differences in their percent of adult emergence, starvation resistance and adult survival when provided with food. Interestingly, females from both cohorts produced diapausing offspring. However, non-diapausing females produced a higher percentage of diapausing offspring than diapausing females, particularly at old ages (26 to 34 days-old). The physiological, behavioral and evolutionary reasons for this are new research questions.

Under our laboratory-controlled conditions and using mass-reared *A. ludens* as hosts, we found that diapause in *D. areolatus* has been maintained after 23 generations, suggesting a genetic component. The developmental time of diapausing individuals ranged from 70 to 281 days, which is shorter than those reported for this species when they were collected in the field in Mexico, Brazil and Argentina (Aluja et al. 1998; Carvalho 2005; Ovruski et al. 2016). These reports included wild individuals coming from different fruit fly species hosts (*A. obliqua*, *A. ludens*, *A. fraterculus*, *A. serpentina*

and *A. striata*), infesting different fruit species (*E. uniflora*, *S. mombin*, *P. guajava* L. and *Tapirira mexicana* March. (Anacardiaceae)), under varied climatic conditions.

Another factor affecting diapause duration is the metabolic reserves of individuals (Ishihara and Shimada 1995; Hahn and Denlinger 2007), because only those larvae with enough nutritional resources will survive this period (Ellers and van Alphen 2002). Despite we found that diapausing individuals had a lower pupal weight, – which could mean lower fat reserves – and adults emerging from diapause were smaller, there were no difference in adult percent emergence between the two cohorts. Aluja et al. (1998) and Ovruski et al. (2016) previously noted the smaller larval and pupal weight of diapausing individuals, but no comparisons had been made on adult dimensions. Wings also were longer in non-diapausing wasps and this could be associated with the greater flight ability observed in this cohort. Kölliker-Ott et al. (2003) proposed that wing size, shape and asymmetry influenced the field fitness in *Trichogramma* egg parasitoids. The changes in body size and fat reserves are among the most notorious characteristics of diapausing individuals, which is related to the energetic demands and fitness costs associated with diapause (Ellers and van Alphen 2002; Hahn and Denlinger 2007).

It is generally agreed that diapause represents a strategy to cope with adverse environmental conditions, including a shortage of hosts. In this context, and contrary to the expectation of an increased starvation resistance in diapausing individuals, our results showed that diapausing and non-diapausing parasitoids had similar survival rates when deprived of food. However, females emerging from diapause and provided with food and water lived longer than non-diapausing females (Table 3, Fig. 4), although difference was not statistically significant. This suggests that adults emerging from diapause could be more efficient using the nutritional reserves.

The higher proportion of female offspring produced by non-diapausing females could be attributed to a lower mating rate in the diapause cohort, thus increasing the production of parthenogenetic male individuals. The higher fecundity of the non-diapausing cohort and the sex ratio biased to females indicate that these parasitoids will show greater population growth rates than diapausing ones. This decrease in reproduction may represent a trade-off for diapause affecting the adult fitness components (Ellers and van Alphen 2002; Hahn and Denlinger 2007; Sadakiyo and Ishihara 2012).

Our most important finding was the effect of mother age of non-diapausing females on the production of offspring entering into diapause. Females of 26 to 34 days old, produced 78% of the offspring that entered into diapause. This suggests that diapause in *D. areolatus* has a hard-wired genetic component and may represent an adaptation to host scarcity in the field (Denlinger 1986). Although the frequency of diapause in *D. areolatus* has been previously associated with the fruit species used by the fruit fly host (Ovruski et al. 2016), in this study, we used the same fruit species (*P. guajava*) and we still found differences between diapausing and non-diapausing individuals respect to maternal age and the proportion of offspring entering into diapause. Some studies proposed that parents might determine whether their offspring

enters into diapause (Denlinger 2002; Hahn and Denlinger 2007). Environmental factors affecting the parental generation may determine the production of diapausing offspring (Saunders 1965). In this sense Gonçalves et al. (2018) showed that temperature influence the immature development and demographic parameters of a Brazilian population of *D. areolatus* parasitizing *A. fraterculus*, but it remains to test whether temperature or other environmental factors affect the frequency of diapause in this species. Our *D. areolatus* population has been exposed to *Anastrepha ludens* larvae in guava fruits under laboratory conditions for 23 generations. During this time, diapausing individuals were systematically sorted out using only non-diapausing individuals to maintain the laboratory colony. Despite the constant environmental conditions and this selection against diapause, the proportion of diapausing individuals did not vary significantly through generations. According to Rahimi-Kaldehy et al. (2018) maternal age together with other abiotic factors (i.e. temperature, humidity, light) can affect the percentage of offspring entering into diapause in *Trichogramma brassicae* Bezdenko, where the percentage of diapause decreases as maternal age increases. An inverse effect was reported for several Pteromalidae parasitoids, such as *Spalangia* sp. and *Nasonia vitripennis* (Walker), where the probability of larvae entering diapause increases if they were born from eggs laid by aging females (Simmonds 1948; Saunders 1965). The same occurred in our study with *D. areolatus*, where the oldest females, emerged from non-diapausing pupae, produced mainly offspring that entered into diapause.

We concluded that diapause in *D. areolatus* has effects on some biological attributes that can be trade-offs in the parasitoids fitness, such as pupal and adult size, flight ability and reproduction. We also found that maternal age has an important effect on the proportion of offspring entering diapause. This represents baseline knowledge to understand how diapause affects the population dynamics of this species and the possible implications in the implementation of the mass rearing and biocontrol applications. The presence of diapausing individuals as part of the offspring of the released population could improve the effectiveness of biocontrol strategies during unfavorable environmental conditions emerging at the optimal time.

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# Two additions to the Iberian myrmecofauna: *Crematogaster inermis* Mayr, 1862, a newly established, tree-nesting species, and *Trichomyrmex mayri* (Forel, 1902), an emerging exotic species temporarily nesting in Spain (Hymenoptera, Formicidae)

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## Abstract

Exotic ants are a growing component of urban and disturbed habitats worldwide. Sampling in two Iberian Mediterranean localities revealed several exotic species. The tree-nesting, black acrobat ant *Crematogaster inermis* Mayr, 1862, has been detected nesting outdoors on a *Robinia pseudoacacia* tree in a public garden in Valencia (Spain) and represents a new addition to the continental European fauna. Collection details, a morphological description, biometry, and the remarkable presence of short spines in smaller specimens are also given for this species. A case of an ephemeral establishment of several exotic ant species in a palm grove is also discussed. This case includes *C. inermis* and *Trichomyrmex mayri*, also a novelty for the Iberian Peninsula.

## Keywords

*Aphis craccivora*, *Crematogaster inermis*, *Robinia pseudoacacia*, *Trichomyrmex mayri*, Spain

## Introduction

Insect communities inhabiting urban and anthropized or degraded habitats are increasingly being enriched by incoming exotic species, usually with the unintentional help of human trade (Aronson et al. 2014; Rabitsch 2010; Sax and Gaines 2003). The Mediterranean regions are especially prone to the arrival of alien biota (Di Castri et al. 1990; Queiroz and Pooley 2018) and the trend seems to be never ending (Seebens et al. 2017; Gaston 2010). The ants, as a group, are among the most ecologically successful organisms and dominate many ecological communities (Wilson 1990). Correspondingly, many well established exotic ants are qualified as pests (Williams 1994; Klotz et al. 2008). Tramp ants (Passera 1994), a subset of the exotics, although, usually remain in an arrested state, never reach pest status. They may be regarded as simple, non-problematic additions to the local fauna. A recent paper by Schifani (2019) is an excellent summary of exotic ants, invasive or otherwise, in Mediterranean Europe. We document several ant species from two localities of the Iberian Mediterranean coast, and which contain exotic novelties for the Iberian myrmecofauna, one also being new for continental Europe.

## Methods

Locality 1: Agost (Alicante, Spain). GPS geographical coordinates 38.4320N, 0.6639W; elevation ca. 325 m a.s.l. A small palm grove (0.1 ha) of *Phoenix dactylifera* L. was surveyed in July 2007 using a visual search for soil surface crawling ants and ants on trees by Dr. Apostolos Pekas. Ants were directly preserved in 70% ethanol, and were identified by one of the authors (KG). The grove was revisited twice (16 Jul. 2016; 26 Apr. 2017). It had been abandoned (as well as the irrigation system) and all visible ant species were collected. In addition, remains from two refuse heaps of *Pheidole* nests were also collected and analysed under the microscope for ant carcasses.

Locality 2. Valencia (Spain). The Jardín del Turia is the biggest urban park (123 ha) in Valencia city (GPS geographical coordinates 39.4823N, 0.3768W; elevation ca. 10 m a.s.l.). Founded in 1986, the vegetation is rich in species, of Mediterranean and exotic origin (Organismo Municipal Autónomo Jardines y Parques de Valencia 2019).

Samplings were carried out in a study to analyse aphid-centred trophic webs inside urban green areas of Valencia (Casiraghi 2019). There were various colonies of *Aphis craccivora* Koch, 1856 on two black locust trees (*Robinia pseudoacacia* L.) (Fabaceae), with one much bigger (DBH: diameter at breast height 39 cm) than the other (DBH 28 cm). Although the trunks were 7 m apart, the canopies of both trees were in contact and we assume all workers to belong to a single colony. The aphids were attended by an unidentified *Crematogaster* ant. The ants were initially collected in May 2019 and preserved in 100% ethanol. The trees were purposely revisited (14 October 2019) to collect more material and to get direct experience of the ant in its habitat (Fig. 1).

One of the authors (XE) identified this species as *Crematogaster inermis* Mayr, 1862. Published morphological and biometrical data on this species are very scarce, so we characterise this local population using taxonomic characters as defined in Longino (2003).



**Figure 1.** *Robinia pseudoacacia* tree (black locust) where *Crematogaster inermis* were nesting (Valencia, Spain). Black arrow indicates the level of nest entrance (Image X. Espadaler).

We measured the first two collected workers plus four of the smallest, four medium and four biggest workers, thus totalling 14 workers. Measurements were made at 60× with a dissecting microscope and are shown as the mean (minimum, maximum) in mm.

- HW** head width; maximum width of head in full-face view, including eyes.  
**HL** head length; perpendicular distance from line tangent to rearmost points of vertex margin to line tangent to anterior most projections of clypeus, in full-face view.  
**SL** scape length; length of scape from apex to basal flange.  
**EL** maximum eye length, measured along maximum diameter.  
**WL** (Weber's length): viewing mesosoma in lateral profile, distance from approximate inflection point, where downward sloping pronotum curves into anteriorly projecting neck, to posteroventral propodeal lobes.  
**ProW** maximum pronotum width in dorsal view.  
**PW** petiole width; maximum width of petiole in dorsal view.  
**PpW** postpetiole width; maximum width of postpetiole, in same view as and perpendicular to postpetiole length.  
**CI** cephalic index= $100 \cdot HW/HL$   
**SI** scape index= $100 \cdot SL/HL$   
**OI** ocular index= $100 \cdot EL/HL$

The species was identified using information from different sources: a) Partial genus revisions of Emery (1926), Santschi (1937), Salata and Borowiec (2015) and Sharaf et al. (2019); b) available keys from Antwiki (2019); c) Type and other images available in Antweb (<http://www.antweb.org> [Accessed on 10 June 2019]), and in The Ants of Egypt ([http://antsafrica.org/ant\\_species\\_2012/antsofegypt/cover.htm](http://antsafrica.org/ant_species_2012/antsofegypt/cover.htm) [Accessed on 10 June 2019]), and d) by direct comparison with identified samples from Egypt kindly donated by Dr. Mostafa Sharaf from Egypt, (reference KG02023 at Antweb) and from Israel. The original description was also checked for general morphological congruence. Vouchers have been deposited in the Museum of Natural History (MNCN, Madrid) and in the Instituto de Biología Integrativa de Sistemas (I<sup>2</sup>SysBio) Centro Mixto Universidad de Valencia-CSIC of Valencia. Remaining workers are in the collection of one of the authors (XE).

## Results

Locality 1. Agost (Alicante, Spain). A total of 17 species were collected by Dr. A. Pekas in 2007, six of them exotic. Three of those exotics (*C. inermis*, *T. destructor* (Jerdon, 1851), and *T. mayri* (Forel, 1902)) were previously unknown in Iberia in 2007. Images and data for *C. inermis* are available at Antweb (KG01956A-1, KG01956A-2 and KG01956A-3; data alluded to by Salata and Borowiec (2019)). *T. mayri* was also the first documented outdoors nesting presence of this species in Europe. In the second (2016) and third (2017) visits we failed to detect any of the several exotic ant species previously observed in 2007 (Table 1). Instead, a cohort of 12 local, native species was already nesting amid the very dry and partially destroyed palm leftovers (Fig. 2).

Locality 2. Valencia (Spain). Two specimens *Crematogaster inermis* were initially collected (5 May 2019; 1 worker; 1 June 2019; 1 worker; A. Casiraghi leg.). A third visit (14 October 2019) and during half-an-hour in a cloudy day with intermittent rain, 27 isolate workers were captured up- or down-coming on the trunk, 25 in one tree and two on a much younger tree. Several *Crematogaster* workers had strongly abraded mandibular dentition, an indication of wood-gnawing behaviour (Fig. 4).

Other ant species present foraging on the trunk of the bigger tree were *Formica* (*Serviformica*) sp., *Lasius grandis* Forel, 1909, and *Pheidole pallidula* (Nylander, 1849). The soil surrounding the base of trees was inspected, although only *Pheidole* and no *Crematogaster* were detected there.

## Description of *C. inermis* worker

HW 1.115 (1.012, 1.200); HL 1.027 (0.962, 1.087); SL 0.836 (0.775, 0.875); EL 0.233 (0.243, 0.250), ProW 0.620 (0.550, 0.700), WL 1.138 (1.050, 1.225); PW 0.349 (0.300, 0.387); PpW 0.304 (0.262, 0.337); CI 108 (105, 110); SI 42 (39, 45); OI 22 (21, 23).

**Table 1.** Ant species present in different years in a date palm grove (Agost, Alicante, Spain).

Ant species	2007	2016–2017
<i>Aphaenogaster iberica</i> Emery	+	+
<i>Camponotus foreli</i> Emery		+
<i>Camponotus micans</i> (Nylander)	+	+
<i>Camponotus sylvaticus</i> (Olivier)		+
<i>Cardiocondyla batesii</i> Forel	+	+
<i>Cataglyphis iberica</i> (Emery)		+
<b><i>Crematogaster inermis</i></b> Mayr	+	
<i>Lasius lasioides</i> (Emery)	+	
<i>Messor barbarus</i> (Linnaeus)	+	+
<i>Messor bouvieri</i> Bondroit	+	+
<i>Monomorium subopacum</i> (F. Smith)	+	
<b><i>Nylanderia jaegerskioeldi</i></b> (Mayr)	+	
<b><i>Pheidole indica</i></b> Mayr	+	
<i>Pheidole pallidula</i> (Nylander)	+	+
<i>Plagiolepis schmitzii</i> Forel	+	+
<i>Solenopsis</i> sp.	+	+
<i>Tapinoma nigerrimum</i> s.l.	+	
<i>Tetramorium biskrense</i> Forel		+
<b><i>Tetramorium lanuginosum</i></b> Mayr	+	
<b><i>Trichomyrmex destructor</i></b> (Jerdon)	+	
<b><i>Trichomyrmex mayri</i></b> (Forel)	+	

2007: irrigated groove; 2016–2017: abandoned groove, without irrigation. In bold, exotic ant species in Spain.



**Figure 2.** Partial view (16 July 2016) of an abandoned date palm grove (Agost, Alicante, Spain) where *Crematogaster inermis*, *Trichomyrmex destructor* and *T. mayri* had been temporary nesting in July 2007 (Image X. Roig).



**Figure 3.** *Crematogaster inermis*, mesosoma lateral view. Bar 0.4 mm. **A** small specimen (HW 1.050 mm) showing small, but visible, triangular propodeal spines **B** medium sized specimen (HW 1.150 mm), with merely angulate propodeum **C** bigger specimen (HW 1.175 mm), with rounded propodeum (Images X. Espadaler).



**Figure 4.** *Crematogaster inermis*. Worker head in frontal view, with abraded mandible denticles. (HW 1.125 mm) (Image X. Espadaler).

Colour deep brown to black. Head slightly wider than long (CI 108), with compound eyes projecting beyond lateral margins in full face view; mandibles longitudinally striate; clypeus not emarginated anteriorly, with rugulose middle area and striated laterals; 6–8 long setae on the anterior clypeal border, directed anteriorly and 1–3 pairs of setae in the central area of clypeus; short, subdecumbent to appressed pubescence all over the cephalic surface, which is mostly glassy smooth, except for longitudinal striae at genae and semicircular striae at the base of antennal insertions; one pair of setae at the level of antennal insertion, and 0–2 pairs on the frontal area; 11 segmented antennae, with a three-segmented club; scape with short decumbent or appressed setae; distal part of scape just reaching the vertex (SI 42). Gula with 2–8 curved anteriorly setae. Occipital carina present.

Pronotum with 0–2 pairs of long humeral setae; mesonotum with a distinct central keel, and broadly angulate postero-laterally, with distinct dorsal face (see Blaimer 2012: 63, fig. 32). Metanotal groove well marked in small specimens to strongly developed in bigger specimens. Smaller specimens with areolate sculpture in the mesoepisternum; in bigger specimens longitudinal rugae are added to the areolate surface. Metapleu-

ron with 10–13 longitudinal rugae. Propodeum variable: in smaller specimens, short spines are developed (Fig. 3A), although they are produced in small angled tubercles in medium sized specimens (Fig. 3B), and absent (Fig. 3C) in bigger specimens. Femorae and tibiae with short appressed pubescence. Petiole, in dorsal view, moderately flared, without dorsoposterior denticles, and postpetiole distinctly bilobed, each of the two segments with one pair of backwards short setae; those setae may be absent but then the basal pit in postero-dorsal position is clearly visible. Gastral tergites without erect setae except for the bordering setae at the posterior border of each segment. Short appressed pubescence over all segments. First gastral sternite with 2–8 curved setae.

## Discussion

Non-cited species for the Iberian Peninsula, collected in 2007, were *Trichomyrmex destructor*, *Trichomyrmex mayri* and *C. inermis*; the three species and all the other exotic ants found have now gone extinct in that locality (Agost, Alicante). Local extinctions, in this case in a man-made palm plantation, are entirely possible, especially if habitat conditions are harshly modified (viz. arrested irrigation). *T. destructor* has been recently collected in a Malaga urban public park (Reyes-López 2019).

The eventual permanence of the documented local, established nests or populations of a majority of exotic ants is only rarely reported. Interestingly, data reported here provide a neat case of a peek-a-boo, a population phenomenon already noted in ants and other organisms, where seemingly well-established populations disappear more or less suddenly for unknown or dubious reasons (Simberloff and Gibbons 2004; Cooling and Hoffmann 2015; Tartally et al. 2019).

A taxonomical revision of this ‘*inermis*’ group may involve an integrative approach (Alpha taxonomy + genetics) and is out of the scope of this paper. While several of those names are likely to be synonyms of *C. inermis*, the present paper is not taxonomic or nomenclatural in scope, and we refrain here to go any further but stating some brief notes on the different forms.

The specific name, *C. inermis*, was reached without difficulty using available keys. Absence of propodeal spines seems to be a rare character state within the genus in the Palearctic, although the clear presence of small spines in the smaller specimens may be a source of confusion if captured as isolates (Fig. 3A–C). This specific polymorphism in propodeal structure, with small spines in smaller specimens, seems to have been overlooked by myrmecologists dealing with the species. Other names in the *inermis* group are *C. fuentei* Menozzi, 1922, *C. warburgi* Menozzi, 1933, and the infraspecific *C. inermis lucida* Forel, 1890, *C. inermis armatula* Emery, 1926, and *C. inermis aphrodite* Santschi, 1937. Those forms are more or less defined based on: i) surface ornamentation and ii) the presence of propodeal angles or very small broadly triangular spines. However, the specific intranidal polymorphism in propodeal profile as shown here (propodeal spines present as a sizer-related trait) may well gauge the morphological

variability of the several above-mentioned names. It is perhaps worth noting here that the supposed Iberian endemic *C. fuentei*, was described and schematically figured (!), as with a “Mesonotum dépourvu de carène” (Menozzi 1922: 327 and fig.1). Although, available images from type material make this doubtful (CASENT0908472).

This species exhibits thermal tolerance between 11 °C and 28 °C and is active day and night in the Negev Desert, although in laboratory settings the critical upper thermal limit is 45 °C (Délye 1968). The species has been categorized as a behavioural dominant that exhibits territorial aggression in the desert of Israel (Segev and Ziv 2012). It is doubtful if this aggressive profile applies to the Spanish population since the climate and other ant species in Valencia are completely different from those in the desert. A dedicated study of ant-ant interactions in Valencia would be necessary to clarify this point. Although typically detected on trees, nests may also be underground (Ofer 2015). The same three-trophic ant-aphid-plant interaction noted in Valencia was already known from Iran (Mortazavi et al. 2015). Feeding habits include items such as scavenging insect corpses, living prey, sap of trees, and honeydew (Délye 1968). *C. inermis* is not categorized as a pest species.

To our knowledge, this is the only free-nesting, outdoors occurrence for *C. inermis* in continental Europe. Data retrieved from antmaps.org (<https://antmaps.org/?mode=species&species=Crematogaster.inermis>) indicates that *C. inermis* and its subspecies, inhabit the Maghreb (Forel 1904, Délye 1968, Cagniant 2005), Egypt (El Bokl et al. 2015), Sudan (Karavaiev 1911), Jordan (Wheeler and Mann 1911), Cyprus (Santschi 1937), Lebanon (Tohmé and Tohmé 2014), Israel (Vonshak and Ionescu-Hirsch 2009), Yemen (Collingwood and van Harten 2001) and reaches Iran (Paknia et al. 2008). The type locality is the Sinai Peninsula (Mayr, 1862). Data from Libya and Syria need verification.

A few exotic *Crematogaster* are known from routine inspection control operations at airports, ports, or quarantine facilities for some countries (USA: Suarez et al. 2005; 15 records from 12 species; 58 years-long database), although not a single *Crematogaster* was detected among the 4355 ant interception records of a 50 year-long database from New Zealand (Ward et al. 2006). Greenhouses and buildings with controlled climate may also provide opportunities to detect exotic *Crematogaster* although, obviously, they do not necessarily represent established nests or free-living, permanent populations.

The genus *Crematogaster* does not seem to provide many instances of established, outdoor-nesting, exotic species, anywhere. Among the few known examples concerning outdoors nests of exotic *Crematogaster* species are those of *C. obscurata* Emery, 1895, nesting in Florida (Deyrup 2007), and *C. scutellaris* (Olivier, 1792) in Germany (Heller 2004) and The Netherlands, where it is “... regularly imported and often established, but is never expanding.” (Boer and Vierbergen 2008). The significance and possible ecological effects of *Crematogaster inermis* from Valencia are very difficult to estimate or interpret (New 2016). We think it is safe not to expect negative consequences of their presence in the gardens of Valencia and that the species will likely remain in the established state, because of the local, climatic conditions, not reaching the spreading, invasive stage (Blackburn et al. 2011), although some occasional spreading can occur to other areas.

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# Phoretic copulation in the velvet ant *Sphaerophthalma pensylvanica* (Lepelletier) (Hymenoptera, Mutillidae): A novel behavior for Sphaerophthalminae with a synthesis of mating strategies in Mutillidae

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## Abstract

Phoretic copulation, a form of phoresy in which a male physically transports a female by flight and/or foot from their initial site of contact before mating, is newly recorded in the Nearctic velvet ant *Sphaerophthalma pensylvanica* (Lepelletier, 1845) (Hymenoptera: Mutillidae). Further, this is the first record of the behavior in the species-rich subfamily Sphaerophthalminae. A description of the *S. pensylvanica* mating observation and photographs are provided. All published observations of copulation events in Mutillidae are critically reviewed in the context of mating strategy, and new terminology is proposed for the mating strategies currently known to occur in the family.

## Keywords

Ethology, phoresy, sexual dimorphism

## Introduction

Velvet ants (Hymenoptera: Mutillidae) are ectoparasitoids of immature holometabolous insects in the orders Hymenoptera, Coleoptera, Diptera, Lepidoptera, and possibly egg predators of Blattodea (Brothers 1989; Brothers et al. 2000). Despite

this wide spectrum of hosts, most host records for mutillids are from solitary bees and apoid wasps (Hymenoptera: Apoidea) (Krombein 1979; Brothers 1989; Brothers et al. 2000; Luz et al. 2016). Extreme sexual dimorphism is the general rule for the family and the sexes have little in common morphologically; males are usually macropterous and the females are always apterous. Sex associations have historically been a major challenge for researchers due to this dimorphism, and the collection of mating pairs in the field, while relatively rare, has been a reliable method for association (Mickel 1937; Nonveiller 1980; Manley and Pitts 2007). Two overarching mating strategies have been observed in Mutillidae: phoretic copulation and in situ copulation.

Phoresy is defined as an interaction between two or more animals in which one individual carries the other(s) for purpose of travel. The individual (or individuals) being carried is termed the phoront(s). Phoresy is particularly common with mites and pseudoscorpions wherein one or a number of individuals will simultaneously travel on a larger arthropod such as a beetle. The carrier animal rarely intentionally carries the phoront except in cases where the phoront is conspecific (or the carrier mistakes the phoront to be conspecific, a common occurrence in Thynnidae (Brown 2000)). Phoretic copulation in Hymenoptera is a form of phoresy in which a larger male physically transports a smaller conspecific female phoront by flight and/or foot from their initial site of contact before mating; the pair may settle on a substrate to mate, or mating may take place during flight (Evans 1969; Brothers 1989). The female is carried by the male primarily by either grasping her around the pronotal neck with his mandibles or by their terminalic union. Phoretic copulation has been observed in three distantly-related families of aculeate Hymenoptera with apterous females: Bethyidae, Mutillidae, and Thynnidae (Evans 1969; Clausen 1976; Brothers 1989; Gordh 1990; Osten 1999; Azevedo et al. 2016). Vivallo (2020) recently reviewed phoretic copulation in aculeate Hymenoptera as a whole with primary emphasis on Thynnidae and the biomechanical aspects of the behavior in that family. For Mutillidae, phoretic copulation has been reported in the following subfamilies and tribes: Dasylabrinae (Dasylabrini), Mutillinae (Ctenotillini, Ephutini, Smicromyrmini, and Trogaspidiini), Myrmosinae (Myrmosini), and Rhopalomutillinae (Table 1). The alternative strategy to phoretic copulation is in situ copulation, where the male does not transport the female from the initial site of contact to mate. These mating strategies in Mutillidae have, thus far, appeared to be representative of taxa at the subfamily and tribe levels. The subfamily Dasylabrinae is the exception wherein both phoretic copulation and in situ copulation have been observed (Table 1).

*Sphaerophthalma pennsylvanica* (Lepeletier, 1845) is a widespread mutillid that occurs throughout the eastern half of the United States, extending as far west as Texas north to Kansas (Krombein 1979). It is one of the most well-studied mutillid species with respect to the parasitoid aspects of its biology (Krombein 1967; Matthews 1997; Pitts and Matthews 2000; Pitts et al. 2010a). Remarkably, there is no published information on its mating behavior. In this contribution, an observation

of phoretic copulation in *S. pensylvanica* is documented and described. Additionally, the published observations of mating strategies in Mutillidae are comprehensively reviewed in order to place this mating observation into the wider behavioral context of the family. This is the first known occurrence of phoretic copulation in Sphaerophthalminae, which is the second largest subfamily of Mutillidae comprising nearly 1,500 described species (Lelej 2005).

## Results

The following observation by J. Roberts of the heretofore undocumented mating behavior of *Sphaerophthalma pensylvanica* occurred on August 3, 2018 in Morgan County, Alabama, along the border of the Highland Rim and Cumberland Plateau regions (Figs 1–4). During a walk through a semi-open deciduous wooded area in late afternoon, what was at first presumed to be a solitary male *S. pensylvanica*, was observed flying from the immediate leaf littered ground to the base branches of a short cedar tree, approximately 9–10 inches (23–25 cm) above the ground. It was when the male attempted to land on these lower twigs/leaves that it was then observed that he dropped a female that he had apparently carried from the leaf litter. The female tumbled a few inches directly below the male and landed on some of the lower twigs/leaves. In an unexpected move, the male immediately descended in a quick flight-assisted scurry to retrieve the female and gripped her firmly behind the head with his mandibles. He once again briefly took flight and carried her higher up into the same small cedar tree to a height approximately 24 inches (61 cm) above the ground. A somewhat blurry, but discernible photo was captured of the moment the male began his descent to retrieve the female after he dropped her (Fig. 1).

Once alighted on the upper twigs/leaves and quickly becoming stabilized, with the male's mandibular grip firm on the pronotal neck of the female, they began copulation at which point it appeared the female began to extrude her stinger which facilitated the coupling of genitalia (Fig. 3). The entire copulative duration was just under two minutes, during which time (and immediately prior to) the male's legs were very active in rhythmic flicking motions, tapping the female on both the metasoma as well as around the gena and pronotum, while alternately tapping the top of her head with the scape of his antennae in the same rhythmic fashion, in between leg tapping. During this process the female did not remain purely passive, but kept a grip on the plant material with her mandibles, fore legs, and mid legs (Figs 3, 4). Toward the end of copulation the female used her hind legs to stroke the mid and hind legs of the male, the purpose uncertain but speculatively could be a tactile communication to the male or simply an attempt to regain footing. Once copulation was complete, the male released the female within moments and promptly flew away, while she quickly climbed downward and eventually scurried back into the leaf litter. There was no post-copula interaction observed between the pair.

## Discussion

### Mating strategies in Mutillidae

This new observation of phoretic copulation in *S. pensylvanica* is recognized as an opportunity to critically review the published information regarding mating strategies in Mutillidae and to develop new terminology that accurately describes them. Data on the mating strategies for 62 mutillid species are comprehensively reviewed in Table 1. References that merely note a pair being collected in copula, or copulating in captivity, were excluded. These observations are numerous in the literature and usually provide no additional information other than the sex association itself. In compiling these data, it became apparent how little is known overall on the mating behavior of the family, especially behavior documented in natural settings. Observations of mating events in captivity have been deemed problematic, as males will attempt to mate with non-conspecific and even non-congeneric females (Ferguson 1962; Manley 1977; Manley and Pitts 2007). Copulation behavior and mating time observed in the laboratory may not be congruent with behavior that would normally occur in the field. The observations cited in Table 1 as being conducted in captivity should be kept with this in mind. The higher classification of Mutillidae in this contribution follows Brothers and Lelej (2017), except *Dolichomutilla* Ashmead, 1899 is considered a member of Mutillini rather than Trogaspidiini, and the two apparent genus-groups that comprise the Mutillini subtribe Ephutina (the *Ephuta* genus-group and the *Odontomutilla* genus-group) are considered full tribes within Mutillinae (Ephutini and Odontomutillini, respectively). These partial modifications in classification are used here in anticipation of a molecular phylogeny of Mutillidae using Ultra-Conserved Elements (Waldren et al. in prep.).

As mentioned previously, there have been two types of mating strategies recognized in mutillids: phoretic copulation and in situ copulation. Two subtypes of phoretic copulation were recognized by Brothers (1989). One was termed “true phoretic copulation” wherein the male initially uses his legs to pick up a female and once terminalic union occurs, phoresy is strictly effected by the genitalia and surrounding metasomal structures; mating occurs during flight or while nectaring. Within Mutillidae, this first subtype is known to occur in the myrmosine tribe Myrmosini and the subfamily Rhopalomutillinae (Table 1). “True phoretic copulation” also occurs in some subfamilies of Bethyridae and Thynnidae (Evans 1969; Osten 1999; Azevedo et al. 2016). The other subtype is known to commonly occur in the subfamily Mutillinae (excluding Mutillini and Odontomutillini) and now in Sphaerophthalminae (*S. pensylvanica*) (Table 1), wherein the female is primarily supported by the male’s mandibular clasp around her pronotal neck, and secondarily by his legs and terminalic union. The pair travels from the initial site of contact by male flight and/or foot and eventually settle on a substrate to finish mating (Nonveiller 1980; Brothers 1989; Brothers and Finnermore 1993). However, this second subtype is technically also “true phoretic copulation,” as the female is carried by the male with his mandibles throughout the mating event, even while the pair are resting on a substrate in copula

**Table 1.** Review of published mating strategy data for Mutillidae.

Taxon	Mating strategy	Size dimorphism	Time in copula	Conditions	Reference	Additional notes
<b>Dasylabrinae: Apteromutillini</b>						
–	–	–	–	–	–	–
<b>Dasylabrinae: Dasylabrini</b>						
<i>Chrestomutilla glossinae</i> (Turner, 1915)	MPC	–	–	in the field and in captivity	Lamborn (1916)	–
<i>Tricholabiodes lividus</i> (André, 1909)	ISC	♂ > ♀	–	in captivity	Bayliss and Brothers (1996)	–
<i>Tricholabiodes thisbe</i> (Péringuey, 1898)	ISC	♂ = ♀	“10–15 seconds”	in captivity	Bayliss and Brothers (1996)	–
<b>Mutillinae: Ctenotillini</b>						
<i>Ctenotilla caeca</i> (Radoszkowski, 1879)†	PC	♂ > ♀	–	in the field	Nonveiller (1963)	–
<b>Mutillinae: Ephutini</b>						
<i>Ephuta floridana</i> Schuster, 1951	PC	♂ > ♀	–	in the field	Deyrup and Manley (1986)	–
<i>Ephuta sabaliana</i> Schuster, 1951	PC	♂ > ♀	–	in the field	Deyrup and Manley (1986)	–
<i>Ephuta slossonae slossonae</i> (Fox, 1899)	MPC	–	–	in the field	Krombein and Norden (1996)	–
<b>Mutillinae: Mutillini</b>						
<i>Dolichomutilla sycorax</i> (Smith, 1855)	ISC	♂ = ♀	“60–100 seconds”	in captivity	Bayliss and Brothers (2001)	–
<i>Mutilla europaea</i> Linnaeus, 1758	ISC	–	a few minutes	in captivity	Drewsen (1847)	–
<i>Mutilla europaea</i> Linnaeus, 1758	ISC?	–	–	in captivity	Hoffer (1886)	–
<i>Mutilla europaea</i> Linnaeus, 1758	ISC?	–	–	in captivity	Su et al. (2019)	–
<b>Mutillinae: Odontomutillini</b>						
–	–	–	–	–	–	–
<b>Mutillinae: Smicromyrmini</b>						
<i>Nemka viduata</i> (Pallas, 1773)	MPC	–	45 minutes (field)	in the field and in captivity	Alicata et al. (1975)	–
<i>Nemka viduata</i> (Pallas, 1773)	PC	–	–	in the field	Matteini Palmerini (1992)	–
<i>Nemka viduata</i> (Pallas, 1773)	MPC	sizes variable	“more than 2 hours”; 45 minutes	in the field and in captivity	Matteini Palmerini (2013)	–
<i>Nemka viduata</i> (Pallas, 1773)	PC	♂ > ♀	–	in the field	Nonveiller (1963)	–
<i>Nemka viduata</i> (Pallas, 1773)	PC	sizes variable	–	in the field and in captivity	Polidori et al. (2013)	mating balls
<i>Nemka viduata</i> (Pallas, 1773)	MPC	sizes variable	“2 h–2 h 15 min” (captivity); “2 h 20 min”; “3 h 7 min”; “2 h 13 min”; “2 h 10 min” (field)	in the field and in captivity	Tormos et al. (2010)	–
<i>Physetopoda balensis</i> (Fabricius, 1787)‡	MPC	♂ > ♀	25 minutes	mating pair collected in the field and observed in captivity	Bertkau (1884)	–
<i>Procecilla decora</i> (Smith, 1879)	MPC	–	“1 hour 22 minutes”	mating pair collected in the field and observed in captivity	Pagden (1934)	–

Taxon	Mating strategy	Size dimorphism	Time in copula	Conditions	Reference	Additional notes
<i>Smicromyrme benefactrix</i> (Turner, 1916)	ISC/PC	–	–	in the field and in captivity	Lamborn (1916)	males attempted female carriage with his mandibles around her pedicel
<i>Smicromyrme jovanovici</i> Nonveiller, 1963§	ISC	♂ = ♀	–	in the field	Nonveiller (1963)	–
<i>Smicromyrme rufipes</i> (Fabricius, 1787)	MPC	–	56 minutes (field); 1 hour 3 minutes (field); 1 hour 10 minutes (captivity)	in the field and in captivity	Crèvecoeur (1930)	–
<i>Sulcotilla</i> sp.	MPC	–	–	museum specimens	Brothers (1975)	–
<b>Mutillinae: Trogaspidiini</b>						
<i>Karlissaidia sexmaculata</i> (Swederus, 1787)	MPC	–	“hours”	in the field	Rothney (1903)	–
<i>Karlissaidia</i> sp. nr <i>sexmaculata</i> (Swederus, 1787)	PC	–	–	museum specimens	O’Toole (1975)	–
<i>Timulla cordillera</i> Mickel, 1938	MPC	–	“approx. 16 hours”	in captivity	Cambra et al. (2018)	–
<i>Timulla dubitata</i> (Smith, 1855)	MPC	♂ > ♀	–	mating pair collected in the field and observed in captivity	Sheldon (1970)	–
<i>Timulla floridensis</i> (Blake, 1879)	PC	♂ > ♀	–	in the field	Deyrup and Manley (1986)	–
<i>Timulla nisa</i> Mickel, 1938	MPC	♂ = ♀	–	in captivity	Cambra and Quintero (1993)	information gleaned from photographs
<i>Timulla oajaca</i> (Blake, 1871)	PC	♂ > ♀	–	mating pair collected in the field	Linsley (1960)	female was supported by male’s legs and genitalic union
<i>Timulla oajaca</i> (Blake, 1871)	PC	–	–	in the field	Hennessey and West (2018)	–
<i>Timulla rufogastra</i> (Lepeletier, 1845)	MPC	♂ > ♀	–	in the field	Bartholomay et al. (2017)	mixed-species mating aggregation
<i>Timulla runata</i> Mickel, 1938	MPC	–	“about 20 hours”	in captivity	Cambra et al. (2018)	–
<i>Timulla suspensa</i> (Gerstaecker, 1874)	MPC	♂ > ♀	–	museum specimens	Bartholomay et al. (2017)	–
<i>Timulla suspensa</i> (Gerstaecker, 1874)	PC	–	–	in the field	Hennessey and West (2018)	–
<i>Timulla vagans</i> (Fabricius, 1798)]	–	–	–	in the field	Fattig (1936)	mating ball
<i>Timulla vagans</i> (Fabricius, 1798)	–	–	“several minutes”	in the field	Shappirio (1947b)	–
<i>Timulla vagans</i> (Fabricius, 1798)	MPC	♂ > ♀	–	museum specimens	Sheldon (1970)	information gleaned from illustration
<i>Trogaspidia (Acutitropidia) aurata</i> (Bischoff, 1920)	MPC	♂ > ♀	–	in the field	Nonveiller (1980)	information gleaned from photograph

Taxon	Mating strategy	Size dimorphism	Time in copula	Conditions	Reference	Additional notes
<i>Trogaspidia</i> ( <i>Acutivropidia</i> ) <i>bugalana</i> (Bischoff, 1920)	MPC	♂ > ♀	–	museum specimens	Brothers (1989)	information gleaned from photograph
<i>Trogaspidia</i> <i>fedtschenkoi</i> (Radoszkowski, 1877)	MPC	♂ > ♀	–	museum specimens	Skorikov (1935)	information gleaned from illustration
<i>Wallacidia melmora</i> (Cameron, 1905)	MPC	–	–	museum specimens	O'Toole (1975)	–
<i>Wallacidia oculata</i> (Fabricius, 1804)	PC	–	–	museum specimens	O'Toole (1975)	venter to venter position
<i>Wallacidia oculata</i> (Fabricius, 1804)	MPC	–	–	in the field	O'Toole (1975)	–
<i>Wallacidia oculata</i> (Fabricius, 1804)	MPC	♂ > ♀	–	in the field	current study (Fig. 7)	–
<b>Myrmillinae</b>						
<i>Myrmilla calva</i> (Villers, 1789)♂	ISC	–	5 to 15 minutes	in captivity	Monastra (1989)	–
<i>Myrmilla erythrocephala</i> (Latreille, 1792)#	ISC	–	just over 20 minutes; roughly for 17 to 19 minutes	in captivity	Monastra (1989)	–
<b>Myrmosinae: Kudakrumiini</b>						
<i>Myrmosula parvula</i> (Fox, 1893)	ISC	–	"14 seconds"	in captivity	Brothers (1978)	–
<b>Myrmosinae: Myrmosini</b>						
<i>Myrmosa atra</i> Panzer, 1801	TPC	♂ > ♀	"9 minutes"; "47 minutes 26 seconds"	in the field	Saxton (2010)	venter to venter position
<i>Myrmosa bradleyi</i> Roberts, 1929	PC	–	–	mating pair collected in the field	Linsley (1960)	–
<i>Myrmosa unicolor</i> Say, 1824	TPC	♂ > ♀	–	mating pair collected in the field	Krombein (1956)	venter to venter position
<i>Myrmosa unicolor</i> Say, 1824	TPC	♂ > ♀	–	museum specimens	Cambra et al. (2018)	–
<i>Myrmosa unicolor</i> Say, 1824	TPC	♂ > ♀	–	in the field	current study (Fig. 6)	–
<i>Myrmosa</i> sp.	PC	–	–	mating pair collected in the field	Pate (1947)	–
<b>Pseudophotsidinae</b>						
–	–	–	–	–	–	–
<b>Rhopalomutillinae</b>						
<i>Bischoffiella cristata</i> (Bingham, 1912)	TPC	♂ > ♀	–	museum specimens	Brothers (1989, 2015)	information gleaned from photograph
<i>Pherotilla oceanica</i> (Mickel, 1935)††	PC	–	–	in the field?	Pagden (1938)	–
<i>Pherotilla rufitincta</i> (Hammer, 1957)	TPC	♂ > ♀	–	museum specimens	Brothers (2015)	information gleaned from photograph
<i>Rhopalomutilla anguliceps</i> (André, 1897)	TPC	♂ > ♀	–	mating pair collected in the field	Brothers (1989)	mating aggregation
<i>Rhopalomutilla clavicornis</i> (André, 1901)	TPC	–	–	mating pair collected in the field	Bridwell (1917)	–
<b>Sphaerophthalminae: Dasymutillini</b>						
<i>Dasymutilla araneoides</i> (Smith, 1862)‡‡	–	–	–	in the field	Manley and Pitts (2007)	mating ball
<i>Dasymutilla araneoides</i> (Smith, 1862)	–	–	–	in the field	Quintero and Cambra (2001)	mating ball

Taxon	Mating strategy	Size dimorphism	Time in copula	Conditions	Reference	Additional notes
<i>Dasymutilla bioculata</i> (Cresson, 1865)	ISC	♂ < ♀	“about twenty seconds”	in captivity	Cottrell (1936)	–
<i>Dasymutilla bioculata</i> (Cresson, 1865)§§	ISC	–	“less than five seconds”	in the field	Manley and Deyrup (1989)	–
<i>Dasymutilla coccineohirta</i> (Blake, 1871)	ISC	–	“a few seconds”	in captivity while in the field	Hurd (1951)	–
<i>Dasymutilla coccineohirta</i> (Blake, 1871)	ISC	–	“2 seconds”	in the field	Manley (1977)	–
<i>Dasymutilla erythrina</i> (Say, 1836)¶¶	ISC	–	“five seconds”	in the field	Linsley et al. (1955)	–
<i>Dasymutilla foxi</i> (Cockerell, 1894)	ISC	–	“over one min on one occasion”	in the field and in captivity	Spangler and Manley (1978)	–
<i>Dasymutilla foxi</i> (Cockerell, 1894)	ISC	♂ = ♀	–	in the field	current study (Fig. 5)	–
<i>Dasymutilla nigripes</i> (Fabricius, 1787)	–	–	“less than 10 seconds”	–	Shappirio (1947b)	–
<i>Dasymutilla nigripes</i> (Fabricius, 1787)	–	–	“a very short period”	–	Shappirio (1947b)	–
<i>Dasymutilla occidentalis</i> (Linnaeus, 1758)	ISC	–	“2 to 5 seconds”	in the field	Tomberlin (1997)	–
<i>Dasymutilla quadriguttata</i> (Say, 1823)	ISC	–	“approximately three seconds”	in captivity while in the field	Remington (1944)	–
<i>Dasymutilla</i> sp.	–	–	“about 30 seconds”	–	Shappirio (1947b)	–
<b>Sphaerophthalminae: Pseudomethocini: Euspinioliina</b>						
–	–	–	–	–	–	–
<b>Sphaerophthalminae: Pseudomethocini: Pseudomethocina</b>						
<i>Calomutilla panamensis</i> Cambra, Brothers, & Quintero, 2020	ISC	–	“35 seconds”	in captivity	Contreras 1993; Cambra et al. (2020)	–
<i>Lophomutilla corupa</i> Casal, 1968	ISC	–	“a minimum of 1 minute 48 seconds and the maximum recorded time was 2 minutes 25 seconds; mean copulation time was 2 minutes”	in captivity	Bergamaschi et al. (2010)	–
<i>Lynchiatilla parana</i> Cambra in: Bergamaschi et al. 2012	ISC	–	“83 seconds and 70 seconds”	in captivity	Bergamaschi et al. (2012)	–
<i>Pseudomethoca frigida</i> (Smith, 1855)	ISC	–	“about 15 seconds”	in captivity	Brothers (1972)	–
<i>Pseudomethoca frigida</i> (Smith, 1855)	–	–	“about fifteen seconds”	in the field	Shappirio (1947a,b)	–
<i>Pseudomethoca propinqua</i> (Cresson, 1865)	–	–	“mating was frequent but brief”	in the field	Jellison (1982)	mating balls
<i>Pseudomethoca pumila</i> (Burmeister, 1854)	ISC	–	“less than one minute, with the maximum time recorded of 58 seconds”	in captivity	Bergamaschi et al. (2011)	–
<i>Pseudomethoca simillima</i> (Smith, 1855)	–	–	“about fifteen seconds”	in the field	Shappirio (1947a,b)	–
<b>Sphaerophthalminae: Sphaerophthalmini</b>						

Taxon	Mating strategy	Size dimorphism	Time in copula	Conditions	Reference	Additional notes
<i>Sphaerophthalma blakeii</i> (Fox, 1893)	ISC	–	“ten to twenty seconds”	in captivity	Ferguson (1962)	–
<i>Sphaerophthalma orestes</i> (Fox, 1899)##	ISC	♂ > ♀	“a few seconds”	in the field	Mickel (1938)	–
<i>Sphaerophthalma pensylvanica</i> (Lepeletier, 1845)	MPC	♂ > ♀	“just under 2 minutes”	in the field	current study (Figs 1–4)	–
<b>Ticoplinae: Smicromyrmillini</b>						
–	–	–	–	–	–	–
<b>Ticoplinae: Ticoplini</b>						
–	–	–	–	–	–	–

† as *Ctenotilla pectinifera* (André, 1893)

‡ as *Mutilla ephippium* Fabricius, 1793

§ *nomen nudum*

| as *Mutilla (Timulla) briaxus* Blake, 1871

¶ as *Myrmilla calva distincta* (Lepeletier, 1845)

# as *Myrmilla erythrocephala bison* (Costa, 1887)

†† as *Rhopalomutilla javana* Pagden, 1938

‡‡ as *Dasymutilla deyrollesi* Mickel, 1937

§§ as *Dasymutilla pyrrhus* (Fox, 1899)

|| as *Dasymutilla clytemnestra* (Fox, 1899)

¶¶ as *Dasymutilla formicalia* Rohwer, 1912

## as *Photopsis salmani* Mickel, 1938

(Nonveiller 1980; Cambra and Quintero 1993; Bartholomay et al. 2017; Cambra et al. 2018; current study). Active transport by flight while in copula is not required for the mating event to be considered “true phoretic copulation.”

In order to accurately characterize these patterns of behavior, new terminology is proposed with respect to Mutillidae to broadly define the two types of mating strategies currently known to occur in the family. **1) Phoretic Copulation (PC)** is a form of phoresy in which a male intentionally carries a female phoront for the majority of their mating event. There are two subtypes of phoretic copulation: **1a) Terminalic Phoretic Copulation (TPC)** is phoresy primarily effected by terminalic union (i.e. the genitalia and surrounding structures) between a male and a female phoront for the majority of their mating event (secondarily with his legs) (Fig. 6). **1b) Mandibular Phoretic Copulation (MPC)** is phoresy primarily effected by a male’s mandibular clasp around a female phoront’s pronotal neck for the majority of their mating event (secondarily with his legs and terminalic union) (Figs 2–4, 7). **2) In Situ Copulation (ISC)** is a non-phoretic mating event that occurs at or near the site of initial contact between a male and a female (Fig. 5).

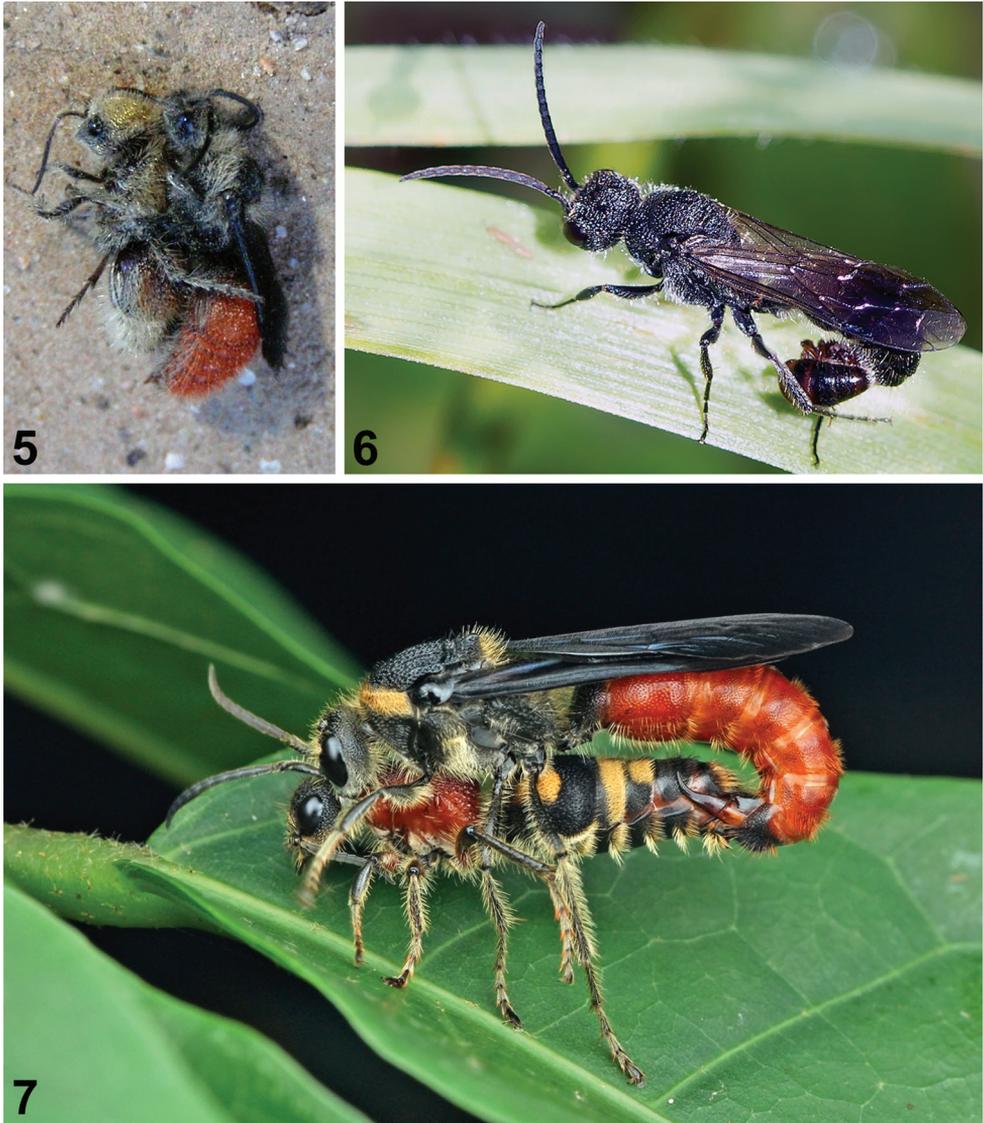
In ISC, there are some observations of males clinging to the dorsum of females during part of the mating event and even clasping their mandibles around the female’s pronotal neck (Cottrell 1936; Ferguson 1962; Bayliss and Brothers 1996, 2001); these events are not considered phoretic copulation as intentional carriage by the male does not occur. This behavior in the context of ISC may play a role in courtship, recognition of conspecificity between the sexes, and/or the biomechanics of mating. Subtypes of ISC may potentially be defined at a later date once more data are available. Mating duration for species that practice PC is often considerably longer than species that practice ISC (Table 1); consequently, mating pairs are collected more often in PC-



**Figures 1–4.** MPC-practicing pair of *Sphaerophthalma pennsylvanica* (Lepelletier, 1845) in Alabama, USA; photographs by Jason D. Roberts.

practicing taxa (Mickel 1937; Nonveiller 1980). The observation described herein for *S. pennsylvanica* is considered MPC.

A potential third subtype of phoretic copulation was described by O’Toole (1975) for the trogaspidiine species *Wallacidia oculata* (Fabricius, 1804) and congeners. As was described: “The posture of copulation in [*W.*] *oculata* is venter to venter, with the male uppermost. The female clings to the sides of the male mesosoma, with



**Figures 5–7.** Examples of each type of mating strategy in Mutillidae **5** ISC, *Dasymutilla foxi* (Cockerell, 1894) in Arizona, USA; photograph by Mark H. Brown **6** TPC, *Myrmosa unicolor* Say, 1824 in New York, USA; photograph by A. D. Levine **7** MPC, *Wallacidia oculata* (Fabricius, 1804) in Southern District, Hong Kong; photograph by ‘aabbabc.’

the tarsal claws gaining purchase on the coarse sculpture of the male.” This mating position is unusual, as most known mating observations in Mutillidae occur with the male venter to female dorsum (although sometimes with wide separation between the male and female’s bodies except for the terminalia). In contrast to this mating posture description, O’Toole (1975) also provided evidence that MPC occurs in *W. oculata* and the now full species *Wallacidia melmora* (Cameron, 1905): “I have seen

several pairs of [*W.*] *o. melmora* in museum collections in which the females are in the mandibular clasp of the males. J. Cardew (personal communication) found a male of [*W.*] *o. oculata* with a female in its mandibles, at Chang Mai, Thailand.” There are two additional published records that describe a venter to venter mating position in the TPC-practicing Myrmosini species *Myrmosa atra* Panzer, 1801 and *M. unicolor* Say, 1824. As detailed in Krombein (1956), both K. V. Krombein and H. K. Townes had independently observed mating pairs of *M. unicolor* in the field that were oriented venter to venter. Additionally, Saxton (2010) observed a mating pair of *M. atra* oriented venter to venter. Prior to the pair’s separation, the couple assumed an end to end mating position and Saxton (2010) determined that the male’s genitalia must have rotated 180° to a facultative strophandrous position (sensu Schulmeister 2001). Male genitalic rotation is also known to occur in the TPC-practicing Thynnidae that engage in male to female feeding (Evans 1969; Vivallo 2020). In contrast to these records, Cambra et al. (2018) included a photograph of a pair of *M. unicolor* that remained in copula after being collected in a Malaise trap which are in a male venter to female dorsum position. An online search for photographs of mating pairs of Myrmosini revealed that females’ bodies are rotated to various degrees with respect to the male. One of these photographs of a mating pair of *M. unicolor* is included here (Fig. 6) and shows a roughly 90° rotation of the female’s body.

For Myrmosini, variable female mating position and likely male genitalic rotation are supported by observations in the field by multiple researchers. For Trogaspidiini, information on venter to venter mating is limited to O’Toole (1975). It is unknown whether this mating posture was observed with live specimens or if it was inferred from museum specimens. If the description in O’Toole (1975) was based on preserved material, the venter to venter posture of the mating pair might be an artifact of how the collector mounted the specimens (and might be how the collector envisaged the posture of the mating pair during the act if they happened to terminate copulation and separate upon being captured). Further, a photograph of a mating pair of *W. oculata* is included in this study (Fig. 7) and they are practicing MPC. We ultimately regard the venter to venter mating position described in O’Toole (1975) as erroneous. All known mating descriptions suggest trogaspidiines practice MPC (Table 1) and the available evidence supports that *Wallacidia* species are no different.

### **The importance of intersexual size dimorphism for phoretic copulation**

Sexual dimorphism in size, with the male being larger than the female, is an important criterion for phoretic copulation to effectively occur (Nonveiller 1963; Deyrup and Manley 1986; Brothers 1989; Tormos et al. 2010; Matteini Palmerini 2013). This size dimorphism is in contrast with other parasitoid Hymenoptera wherein females are commonly larger than males (Charnov et al. 1981; O’Neill 1985; Hurlbutt 1987; van den Assem et al. 1989). In some taxa that are known to normally practice MPC, some

male individuals are similar or smaller in body size to the female they are mating with and are physically unable to transport her by flight or even by foot; facultative ISC consequently occurs (Nonveiller 1963; Alicata et al. 1975; Deyrup and Manley 1986; Tormos et al. 2010; Matteini Palmerini 2013; Polidori et al. 2013). It is unknown if the reverse situation also occurs wherein a species that normally practices ISC due to similarity in male and female size might practice facultative MPC with unusually large males. In evidence against the latter situation, Cottrell (1936) observed that for *Dasymutilla bioculata* (Cresson, 1865), a sphaerophthalmine species that practices ISC, larger males were mechanically unable to copulate with smaller females. Females are often larger than males in this species, and mating was successful when smaller males mated with larger females. Additionally, male aptery and brachyptery, which are uncommon in Mutillidae (Cambra and Quintero 2007, 2017), would limit phoretic copulation by flight but not by foot; mating behavior for species with flightless males has yet to be observed, though. The cause of adult intra- and intersexual size differences within a mutillid species is primarily predicated upon host choice.

Mutillids are generally solitary ectoparasitoids that may parasitize more than one host species. It has long been known that the size of the host determines the size of the adult mutillid, which explains the common occurrence of adult size variation (Mickel 1924; Deyrup and Manley 1986; Brothers 1989; Hennessey 2002). If a female mutillid parasitizes more than one host species that vary in size in relation to one another, her offspring will consequently vary in size. In some mutillid taxa, one sex is on average larger than the other, and the underlying mechanics for sex allocation in mutillids remained unknown until relatively recently. Of critical relevance to the new discovery of phoretic copulation in *S. pensylvanica* is an investigation into sex allocation in this species by Pitts et al. (2010a). Their results supported facultative size-dependent sex allocation in which males typically develop from larger hosts and females develop from smaller hosts. Due to the sex-determination system of haplodiploidy in Hymenoptera, female *S. pensylvanica* are able to choose whether to oviposit a fertilized or unfertilized egg onto a specific host. Unfertilized eggs, which develop into males, are more often deposited on larger hosts, such as the organ pipe mud dauber *Trypoxylon politum* (Drury, 1773) (Hymenoptera: Crabronidae); female eggs are usually deposited on smaller *Trypoxylon* species and other taxa (Matthews 1997; Pitts et al. 2010a). Pitts et al. (2010a) concluded that female *S. pensylvanica* likely use host body length and/or nest diameter as criteria for which sex of egg—male or female—to oviposit on a host rather than the criterion of host mass. The difference in size between the male and female mating pair of *S. pensylvanica* documented herein is substantial (Figs 2–4), and the size dimorphism prerequisite for phoretic copulation is clearly met. Although a rare occurrence, female *S. pensylvanica* have been reared from *T. politum* and males reared from smaller *Trypoxylon* species (Pitts et al. 2010a). More mating observations are necessary for *S. pensylvanica* to see how mating is carried out, if at all, between these smaller males and larger females. Facultative size-dependent sex allocation is likely widespread among PC-practicing mutillids due to the importance of intersexual size dimorphism.

## Phoretic copulation in Sphaerophthalminae

The genus *Sphaerophthalma* Blake, 1871 is a paraphyletic assemblage of 81 described species classified into 17 species-groups (Pitts et al. 2010b; Pitts and Sadler 2015). *Sphaerophthalma pensylvanica* (Lepelletier, 1845) is currently placed in the *S. pensylvanica* species-group along with *S. auripilis* (Blake, 1871), *S. boweri* Schuster, 1944, and *S. nocticaro* Pitts, 2005 (Pitts and Sadler 2015). Given that these other members of the species-group also show the same differences in body size between the sexes, it is likely that they practice MPC as well. Unfortunately, the females of most of the remaining *Sphaerophthalma* species, as well as the related large genera *Photomorphus* Viereck, 1903 and *Odontophotopsis* Viereck, 1903, are unknown. The known females are closer in size to the males and there seem to be no other likely candidates for MPC in *Sphaerophthalma* outside of the *S. pensylvanica* species-group or the related genera *Photomorphus* and *Odontophotopsis*.

There are a few unusual distributions in Sphaerophthalminae that might be due to dispersal via PC. Sphaerophthalmines primarily occur in the Nearctic, Neotropical, and Australasian regions, with two small genera occurring in the Palearctic (Europe, China, Japan, Republic of Korea) and Oriental (China, Taiwan) regions. These latter two genera, *Cystomutilla* André, 1896 and *Hemutilla* Lelej, Tu, & Chen, 2014 were recently reviewed by Tu et al. (2014). Molecular data has revealed that *Cystomutilla* is closely related to the nocturnal Nearctic Sphaerophthalminae (Waldren et al. in prep.). The practice of phoretic copulation, which has, in part, been hypothesized to aid the apterous females in traversing physical barriers such as water (Evans 1969), is not out of the realm of possibility in *Cystomutilla* and *Hemutilla* in light of the behavior being discovered in *S. pensylvanica*. Another genus in which PC may have played a role in dispersal is the primarily Australian genus *Ancistrotilla* Brothers, 2012. Several species are known to occur in New Caledonia and one in Vanuatu, an archipelago of volcanic origin (Brothers 2012; Lo Cascio 2015). The only species known so far from both sexes, *Ancistrotilla azurea* Brothers, 2012, which occurs in Vanuatu, meets the size prerequisite for phoretic copulation with males being larger than females. Additionally, the single known female was apparently collected in the same Malaise trap as fifteen males and could potentially have been carried into the trap by a male.

## Conclusion

Based on prior knowledge, it was thought that mating strategies in Mutillidae were confined to the family-group levels of subfamily, tribe, or subtribe (Table 1). Members of the subfamily Sphaerophthalminae were previously known to only practice ISC. With the discovery of MPC in *S. pensylvanica*, it is revealed that membership to a higher taxon is not always reliable for predicting a species' mating strategy. Ironically, *S. pensylvanica* is the type species of *Sphaerophthalma* Blake, the genus from which the subfamily name Sphaerophthalminae is derived. As this is the only known mating observation for this species and species-group, more information is needed to determine the consistency of this behavior especially with respect to intersexual size

variation. Additional fieldwork is also necessary to get a better idea of how prevalent PC is in Sphaerophalminae. Respecting the historical challenge of discovering mating mutillid pairs in the field, male morphology combined with consistent interspecific size differences in a species could be used as preliminary lines of evidence for the practice of phoretic copulation.

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# Observations of neotropical social wasps (Hymenoptera, Vespidae) preying on eggs and tadpoles of the frog *Engystomops pustulosus* (Amphibia, Leptodactylidae)

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## Abstract

In Trinidad, West Indies the social wasps *Angiopolybia pallens* and *Polybia rejecta* come to foam nests of the frog *Engystomops pustulosus*, where they prey on eggs and tadpoles. Frog offspring in early-stage foam nests appear to be almost immune to predation by the wasps, but they become more vulnerable as the nests age and lose definition.

## Keywords

*Angiopolybia pallens*, *Engystomops pustulosus*, *Polybia rejecta*, predation, túngara frog

## Introduction

*Engystomops pustulosus* (= *Physalaemus pustulosus*) (Cope) is a small, nondescript neotropical frog ranging from Mexico to northern South America (Rand 1983). In Trinidad, West Indies, it is one of six leptodactylids that lay their fertilized eggs into viscous foam nests (Murphy et al. 2018). Breeding takes place in very small, shallow, temporary pools.

As the eggs are laid and fertilized, the female secretes a viscous, proteinaceous fluid, which the male beats into a foam with his hindlegs (Breeder 1946; Dalgetty and Kennedy 2010; Heyer and Rand 1977; Ryan 1985), rather like beating egg whites in the kitchen.

This mass of white foam typically floats on the water surface, enveloping the eggs. The tadpoles may remain in the nest for up to five days before breaking through to the water below (Rand 1983). Foam nests of *E. pustulosus* can be distinguished from those of sympatric species in Trinidad by their placement, size and form (Murphy et al. 2018:113–123), so that they almost always can be identified with confidence even in the absence of adult or immature frogs. They disintegrate over time, first becoming flatter and then losing their integrity. The speed of this process evidently varies according to external conditions. For example, heavy rain hastens the process (Ryan 1985).

Adult *E. pustulosus* are taken by a number of predators (Rand 1983; Ryan 1985). Much less is known of the immature stages' natural enemies. The foam nest has no discernible taste (pers. obs.), but it is presumed to provide substantial physical protection to the eggs and the tadpoles as long as these latter remain inside it. Where the larger frog *Agalychnis callidryas* (Cope) is present, its tadpoles often eat *E. pustulosus*'s eggs (Heyer and Muedeking 1976). Once out of the nest, the tadpoles are evidently vulnerable to a variety of predators, including tadpoles of the larger frog *Leptodactylus pentadactylus* (Laurenti) (Heyer and Muedeking 1976). The adults are often preyed upon by the bat *Trachops cirrhosus* (Spix) (Gomes and Reid 2015; Ryan 1985). Among

*Angiopolybia pallens* (Lepelletier) is a swarm-founding social wasp that ranges from Panama south to Peru and central Brazil (Richards 1978:233). Accordingly, it is broadly sympatric with *E. pustulosus* in northern South America and part of Central America. In Trinidad it is evidently the most abundant social wasp in forests (pers. obs.). Another swarm-founding wasp, *Polybia rejecta* (F.), has a similarly broad distribution (Richards 1978:122), where it is similarly abundant in more open habitats. Both species forage on a broad variety of resources, including carrion (O'Donnell 1995), at which *A. pallens* is especially prevalent (Silveira et al. 2005; pers. obs.). *P. rejecta* is known to prey on the eggs of *A. callidryas* (Warkentin 2000).

## Methods

On 12 May 2018 we noticed several female *A. pallens* landing on *E. pustulosus* foam nests in the Arena Forest Reserve (10°33'N, 61°13'W) in central Trinidad. The habitat is mature secondary rain forest on rolling hills watered by many streams. The wasps appeared to be burrowing into the nests to a shallow depth. On 23 May we returned for further observations.

All nests were in potholes in a degraded paved road. For our purposes, it is convenient to recognize three states of the completed nest. In stage 1, the nest is coherent and domed, commonly to 2–4 cm above the water, as when freshly produced (Fig. 1a). In stage 2, it remains coherent but is flattened throughout to no more than 1 cm (Fig. 1b), thereby increasing its surface/volume ratio. And in stage 3 it is distinctly losing its integrity (Fig. 1c).



**Figure 1.** Stages in the disintegration of *Engystomops pustulosus* foam nests. **a** Stage 1; fresh, domed above the water **b** Stage 2; distinctly flatter, but still coherent **c** Stage 3; very flat and losing coherence.

**Table 1.** Pools in a degraded paved road in Trinidad and their complements of *Engystomops pustulosus* foam nests. See text for explanation of nest stages. One of the stage-2 nests in pool no. 1 was evidently an amalgamation of several original nests from different pairs of frogs.

Pool no.	Stage 1	Stage 2	Stage 3	Wasp visits?
1	4	4	6	+
2	4	4	0	–
3	0	1	0	–
4	1	0	0	–
5	1	1	2	+
6	1	0	0	+
7	0	3	0	+

We made observations at seven pools varying in their composition of foam nests (Table 1). Pool no. 1 was exceptionally large with a surface area of roughly 1 m<sup>2</sup>. At this and several other sites along the road we found wasps landing on the nests. Most of the wasps were *A. pallens*, with only a very few *P. rejecta*, so that all observations below are from the former species except where noted. Our attention was first draw to several *A. pallens* atop two stage-1 nests at a site with no other nests.

## Results

Paring away three stage-1 nests from above, we found abundant dispersed eggs, with the outer 5 or 6 mm free of eggs. Exposed eggs were readily taken by wasps when we stepped away. Some wasps remained to eat the eggs on the spot, while others flew away with them.

Our preliminary observations had been of wasps at a pool with only stage-1 nests. Where nests of different stages were found together, the wasps showed a distinct preference for stage-2 nests (Table 2). This preference is readily explained. Unlike in stage-1 nests, tadpoles were commonly visible just below the surface film of the much flatter nests. They tended to be made even more conspicuous by their movement. Stage-3 nests presumably had nothing to offer except foam.

Wasps often fluttered their wings while walking over the nest surface, possibly as a way of avoiding becoming caught in the surface film. While *A. pallens* commonly burrowed to a depth of its entire head and part of its thorax (Fig. 2), *P. rejecta* hardly penetrated below the surface in our observations. We observed several captures from stage-2 nests, but none by either species from a stage-1 nest except when we exposed eggs.

A few times we saw *P. rejecta* carrying away fragments of foam with no evident egg or tadpole. Lacey (1979) reported *A. pallens* collecting not only eggs and tadpoles but the foam itself from foam nests of *Leptodactylus pentadactylus*. However, in our observations any collection of foam from *E. pustulosus* nests was at most a very occasional activity, not at all like the large numbers of wasps that gather at fresh fruitfalls (pers. obs.) This suggests that nest foam is of only marginal nutritional value and was only taken by wasps that failed in the search for eggs or tadpoles.

**Table 2.** Wasp landings on *Engystomops pustulosus* foam nests in pool 1. Further explanation in text.

	Stage 1	Stage 2	Stage 3
<i>Angiopolybia pallens</i>	3	50	0
<i>Polybia rejecta</i>	5	15	0

**Figure 2.** *Angiopolybia pallens* burrowing into an *Engystomops pustulosus* foam nest.

## Discussion

While characterizing social wasps as “opportunistic, generalist prey foragers”, Raveret-Richter (2000) notes that individuals may return repeatedly to sites of earlier hunting success, making them facultative specialists. *E. pustulosus*'s foam nests are large, very conspicuous (white against the mainly dark forest floor) and stationary. Accordingly, any wasp that has learned to access the abundant eggs and/or tadpoles has good reason to specialize on any nest that she encounters.

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