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How a slow-ovipositing parasitoid can be used successfully as a biological control agent against the invasive mealybug *Phenacoccus peruvianus:* implications for future classical conservation and biological control programs.

Beltrà, A.[1]; Soto, A.[1]; Tena, A.[2]

[1]*Instituto Agroforestal Mediterráneo, Universitat Politècnica de València; Camí de Vera s/n; 46022 València, Spain*

[2]*Unidad Asociada de Entomología UJI-IVIA-CIB CSIC. Centro de Protección Vegetal y Biotecnología; Instituto Valenciano de Investigaciones Agrarias, IVIA; Ctra. de Moncada a Náquera km 4.5; E-46113-Moncada (Spain).*

* Corresponding author. Tel: (+34) 96 387 92 52

E-mail address: asoto@eaf.upv.es

Abstract

Acerophagus n. sp. *near coccois* (Hymenoptera: Encyrtidae) is the major parasitoid of the invasive mealybug *Phenacoccus peruvianus* (Hemiptera: Pseudococcidae) in eastern Spain and could provide a successful biological control of this pest. The success of mealybug parasitoids relies on their ability to overcome the hosts' defenses and to parasitize in ant-tended patches. In this work we therefore studied the tritrophic interactions among mealybugs, their parasitoids and tending ants to determine the ability of *Acerophagus* sp.to control *P. peruvianus* in eastern Spain and to establish efficient biological control programs for this mealybug in areas with different environmental conditions. Specifically, we examined *Acerophagus* sp. oviposition behavior and handling time, *P. peruvianus'* defensive responses and the influence of tending ants on parasitism in urban landscapes. In nearly all the cases studied *Acerophagus* sp. successfully parasitized the hosts regardless of their size and did not host-feed. On the other hand, *Phenacoccus peruvianus* used three different strategies as a response to oviposition: wriggling, swiveling around the inserted stylet and walking away. Host defense significantly reduced parasitism rates and the smaller mealybugs showed better defensive behavior. Handling time was not influenced by the host defense but was a time-consuming process that required more than 30 minutes. The presence of tending ants reduced the time spent by parasitoids in the patch and disrupted oviposition. The low numbers of ants observed tending mealybug colonies in Spanish urban landscapes could explain why this parasitoid, with a long handling time, is an efficient biological control agent for *P. peruvianus*.

Keywords: biological control, host defense, handling time, ants, urban landscapes, parasitoid behavior.

1. Introduction

Hemipteran honeydew producers are sap-feeding insects with clumped sedentary habits (Byrne and Bellows, 1991; Gullan and Kosztarab, 1997; Robert, 1987). Their exposure and poor mobility make them particularly vulnerable to parasitism and predation and they have consequently developed diverse defensive strategies. They hide from their natural enemies by adopting cryptic behavior and reducing chemical cues (Godfray, 1994; Gross, 1993; Vet and Dicke, 1992). When these defense mechanisms fail they use morphological and behavioral tactics, such as protective coverings, evasive and aggressive movements or defensive secretions to avoid predation or oviposition (Godfray, 1994; Gross, 1993). Additionally, honeydew producers have developed mutualistic relationships with ants, which feed on the excreted honeydew and in exchange provide them with protection against their natural enemies (Gullan, 1997; Way, 1963; Weiss, 2006).

Most of these defensive strategies have also been adopted by mealybugs (Hemiptera: Pseudococcidae). Several species seek refuge in vine bark crevices, sugarcane leaf sheaths or citrus sepals to escape parasitism and predation (Bartlett, 1978; Berlinger and Golberg, 1978; Daane et al., 2006; Franco et al., 2009; Moore, 1988). The role of the mealybugs' waxy cover in their interactions with natural enemies is not yet clear. It is believed that the cover might provide protection against predators (Foldi, 1983; Gullan and Kosztarab, 1997). However, this mechanism has not been so clearly described as in other scale insects with harder covers, such as soft (Hemiptera: Coccidae) and armored scales (Hemiptera: Diaspididae) (Daane et al., 2000; Gullan and Kosztarab, 1997; Honda and Luck, 1995; Lampson et al., 1996; Tena and Garcia-Marí, 2008). On the other hand, the absence of a sclerotized cover allows mealybugs to carry out evasive movements similar to those performed by aphids (Hemiptera: Aphididae) (De Farias and Hopper, 1999; Wyckhuys et al., 2008). In fact, some of these movements, such as wriggling, swiveling around the stylet, getting up, walking away, and secreting defensive exudates, have been recorded to thwart parasitoid oviposition (Bartlett, 1961; Boavida et al., 1995; Bokonon-Ganta, 1995; Bugila et al., 2014a; Bynum, 1937; Cadèe and van

Alphen, 1997; Pijls, 1995). Like other honeydew producers, mealybugs establish mutualistic relationships with ants that interfere with biological control (Daane, 2007; González-Hernández et al., 1999; Mgochecki and Addison, 2009; Pekas et al., 2010; Tena et al., 2013). Among the benefits that mealybugs obtain from tending ants are honeydew removal, transport and shelter, as well as protection from their natural enemies (Gullan, 1997). Ants disrupt predation and parasitism and their action can be enhanced by some of the aforementioned defensive mechanisms if these increase handling times, becoming defensive synergic strategies (Bartlett, 1961; Barzman and Daane, 2001).

Ant attendance modifies the relationship between honeydew-producing species and their natural enemies. Some natural enemies have developed mechanisms to overcome ant attendance and have become successful as mealybug biological control agents. Lacewings (Neuroptera: Chrysopidae), coccinellids (Coleoptera: Coccinellidae) and predatory silver flies (Diptera: Chamaemyiidae) have evolved behavioral, chemical and physical strategies to become furtive predators. Some species such as *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) and *Ceraeochrysa cincta* Schneider (Neuroptera: Chrysopidae) mimic their prey to avoid detection by ants (Daane et al., 2007; Eisner and Silberglied, 1988; Majerus et al., 2007). Some parasitoids paralyze mealybugs to overcome active defenses, reduce handling times and elude aggressive tending ants (Bartlett, 1961; Barzman and Daane, 2001; Gross, 1993; Pennachio and Strand, 2006; Zain-ul-Abdin et al., 2013).

The *Phenacoccus peruvianus* Granara de Willink mealybug (Hemiptera: Pseudococcidae) is an invasive species of Neotropical origin that was detected in the Mediterranean Basin in 1999 and has recently been found in California (under eradication) (Beltrà et al., 2010; Arakelian, 2013). It is a polyphagous insect that feeds on several ornamental plants, such as *Bougainvillea spp.*, *Myoporum laetum*, and *Aucuba japonica*, and is a serious pest in urban landscapes and ornamental nurseries. A recent study shows that *P. peruvianus* has been fortuitously controlled by a new species of genus *Acerophagus* Smith in eastern Spain (Hymenoptera: Encyrtidae) (Beltrà et al., 2013b). This parasitoid

of unknown origin was first recorded in 2008 and has displaced the native parasitoid *Leptomastix epona* Walker (Hymenoptera: Encyrtidae) as the most abundant biological control agent of *P. peruvianus*. *Acerophagus* sp. is a koinobiont, parthenogenetic and facultative gregarious parasitoid (Beltrà et al., 2013c). These biological traits, together with the use of small young mealybugs as hosts for the females to lay eggs, explain the high potential of *Acerophagus* sp. as parasitoid of *P. peruvianus.* To date it has behaved as a host-specific parasitoid (personal observations) and has become an interesting candidate for classical biological control in new areas where *P. peruvianus* has appeared.

The success of mealybug parasitoids depends on their capacity to overcome their hosts' defenses and to parasitize species tended by ants (Bartlett, 1978; González-Hernández et al., 1999; Gross, 1993; Moore, 1988). The identification of these interactions is crucial to understanding the success of *Acerophagus* sp. in controlling *P. peruvianus* in eastern Spain and to establish efficient biological control programs of this invasive mealybug in other areas with different environmental conditions. With this aim in view, we i) describe the oviposition behavior of *Acerophagus* sp. and the defensive responses of its host *P. peruvianus*, ii) assess the influence of defensive responses on parasitoid handling time and parasitism, and iii) describe and evaluate the effect of ant attendance on *Acerophagus* sp. oviposition.

2. Materials and methods

2.1. Mealybug and parasitoid colonies

P. peruvianus was cultured at the Entomology Department of the Polytechnic University of Valencia (Valencia, Spain) using specimens collected from *Bougainvillea glabra* plants on the university campus. The mealybugs were reared on sprouting organic potatoes inside plastic sandwich

boxes (16.5 x 11 x 6 cm) with a 6.5 cm diameter aperture covered by a 0.2 x 0.2 mm muslin mesh for ventilation and kept in a dark environmental chamber at 25 ± 2°C and 65 ± 10 % RH.

The culture of *Acerophagus* sp. was based on specimens emerged from *P. peruvianus* mummies collected from *B. glabra* plants on the campus and reared on *P. peruvianus* specimens from the laboratory culture under the conditions described above (Beltrà et al., 2013c). To obtain newly emerged adult parasitoids for our experiments, mealybug mummies were gently transferred into 10 x 1.5 cm vials topped with a plastic lid with a central hole covered with muslin to allow ventilation and a streak of honey on the inner wall. These vials were kept in an environmental chamber at 25 ± 2°C and 65 ± 10 % RH and 14:10 h L/D photoperiod and were checked daily from 8.00-10.00 for adult emergence. If gregarious parasitoids emerged from the same mummy, they were separated and placed individually in new vials for use in the assays. For our experiments, we used unmated 3 to 5-day old *Acerophagus* sp. females since they reproduce parthenogenetically.

2.2. Laboratory behavioral observations

The oviposition behavior of *Acerophagus* sp. and the defensive responses of its host *P. peruvianus* were evaluated by direct observations in open arenas, which consisted of an open 5.3-cm diameter Petri-dish in which a leaf-disk (Ø 5 cm) of *Aucuba japonica* was placed upside-down over a layer of 8 g/l Bacteriological agar (Karamaouna and Copland 2000). A single mealybug was gently transferred from the culture to each arena using a wet camel-hair brush 24 hours before the assay. Mealybug length was measured with a stereoscope provided with an ocular micrometer. First nimphal instars (< 0.5 mm) not susceptible to parasitism were excluded (Beltrà et al., 2013c). A female parasitoid was then placed in the arena and both parasitoid and mealybug behaviors were observed under a compound microscope illuminated with cold light. The recording started when the parasitoid came into contact with the mealybug and ended when the parasitoid left the arena for more than two minutes due to host rejection or after oviposition. The frequency and time spent by

the parasitoid and the host in each behavioral event were recorded by JWatcher 1.0 software (Blumstein and Daniel, 2007) and the experiment was replicated 81 times.

Parasitoid action patterns were classified as follows: encounter – encountering the host after foraging; antenna drumming – drumming on the body of the host with its antennae; ovipositor tap – assessing the host by tapping the body with its ovipositor; rest – resting motionless in front of the host; grooming – cleaning its body (antenna, legs, ovipositor and wings); probing – probing the host's body with the ovipositor; abdominal movements – circular abdominal movements performed by the parasitoid with the ovipositor inside the host's body; abdominal contact – placing the ovipositor completely inside the host until the parasitoid abdomen contacts the host; end of the encounter – leaving the patch after concluding oviposition, refusing parasitism, or a successful mealybug defense. Mealybug defensive action patterns were classified following Boavida et al. (1995): wriggling – rough up-and-down abdominal movements; moving – swiveling around the inserted stylet; getting up – getting up, withdrawing the stylet and walking away. We considered that a mealybug defended itself from an oviposition attempt when it carried out at least one of the above behaviors.

After the observation period, the parasitoid was removed and the petri dish was closed by a lid with two 1 cm diameter holes covered by a muslin mesh to permit ventilation, sealed with Parafilm® (Structure Probe, Inc., West Chester, PA) and placed in an environmental chamber at 25 ± 1°C and 65 ± 10 % HR and 14:10 h L/D photoperiod. Ten days later we confirmed whether or not the mealybugs were parasitized (i.e. mummified) and those without signs of parasitism were dissected to determine whether they contained encapsulated parasitoid eggs (Beltrà et al., 2013c).

2.3 Parasitoid residence time and behavior on ant-attended patches

To test the effect of ants on the searching behavior of *Acerophagus* sp., its residence time and oviposition attempts were recorded in tended and non-tended colonies of *P. peruvianus.* For this, female parasitoids from the laboratory culture were individually released in *B. glabra* bracts

with more than ten mealybugs. This test was performed in an urban green space in the city of Valencia (Spain) (39.476767N, 0.341123W) with ten *B. glabra* climbing plants and intense *Lasius grandis* Forel (Hymenoptera: Formicidae) ant foraging activity. Bracts of *B. glabra* were used as patches as mealybugs show a strongly clumped distribution in these plant strata (Beltrà et al., 2013a). Prior to parasitoid release, the patches were observed for five minutes and were classified as tended by ants when at least one ant visited the colony. After releasing the parasitoid, we recorded by visual observation the time it spent in the patch, any oviposition attempts and disruptions by ants for up to 30 minutes. A total of 20 observations were accomplished for tended and non-tended colonies. The observations were carried out between 14th June and 17th July 2013 between 10:00 am and 13:00 am.

2.4 Survey of Phenacoccus peruvianus *ant-attended colonies*

Fifteen urban green spaces on the Mediterranean coast of Spain and France were monitored from 10.00 to 13.00 hours in June and July 2013. The sampling sites had an average surface of 1 ha with more than 10 mature climbing *B. glabra* and/or hybrid *B.* x *buttiana* plants. We looked for *P. peruvianus* colonies (one or more mealybugs) for a maximum of 5 minutes per plant or until 20 colonies were found per plant and if tended by ants the ant species was recorded. A minimum of 10 plants and a maximum of 160 colonies were observed in each sampling site. *Phenacoccus peruvianus* and *Acerophagus* sp. (Beltrà et al., 2013a; Beltrà et al., 2013b) as well as *L. grandis* (Paris and Espadaler, 2009; Pekas et al., 2011) are abundant during these months.

2.4 Statistics

ANOVA was used to check for differences on the time spent by the parasitoid on each attacking behavioral event [we pooled the events carried out during oviposition: probing, abdominal movements and abdominal contact]. We also employed linear models assuming normal error

variance to study the influence of the mealybug defense and length on parasitoid handling time. Data were normalized by logarithmic transformation when required.

We also used generalized linear modeling techniques assuming binomial error variance to evaluate: i) the influence of mealybug size on its predisposition to defend itself against the parasitoid; ii) the effect of mealybug defense and size on parasitism; and iii) the influence of ants on the capacity of the parasitoid to remain in a patch more than 30 minutes and attempt oviposition. All the analyses were performed using the R statistical software (R Development Core Team, 2011) and the Sarkar 2008 package lattice .

3.Results

3.1. Parasitoid behavior

The oviposition behavior of 81 parasitoids was examined directly for 34 hours under a microscope. *Acerophagus* sp. were seen to walk randomly around the arena drumming with their antennae on the leaf surface. Any encounters always started with antennal contact (Fig. 1). After the encounter, the parasitoids spent some time assessing the host with their antennae or tapping it with the ovipositor. This behavior alternated with periods of grooming and resting. They rejected hosts before probing only on two occasions out of 81. When probing, they drilled a hole in the mealybug's body and inserted the whole ovipositor until its abdomen contacted the host body when they parasitized large insects. However, they did not insert the whole ovipositor in small mealybugs (second nymphal instar) because the ovipositor was longer than the host. After probing, they performed circular abdominal movements. There was a positive correlation between these movements and successful oviposition in 27 of the 38 cases studied ($\chi 2$= 18.40; df = 1; P < 0.0001). Additionally, we did not observe any host feeding attempts.

When we analyzed the time series of successful ovipositions, the events that occurred from host encounter until probing were significantly shorter than oviposition (ANOVA, F = 117.61; df = 4,

158; $P < 0.0001$) (Fig. 2). Specifically, the parasitoid dedicated 0.66 ± 0.09 min and 1.47 ± 0.32 min to drumming the host with its antenna and tapping it with its ovipositor, respectively. It also spent 0.67 ± 0.15 min grooming and 0.75 ± 0.14 min resting while assessing its host. On the other hand, longer times were spent on oviposition events: probing, abdominal movements and abdominal contact (33.33 ± 4.41 min).

3.2. Mealybug defensive behavior and parasitism

Overall, 66.67 ± 5.27% mealybugs (54 out of 81) defended themselves from parasitoid attack. The commonest defensive response of *P. peruvianus* during the oviposition process was a sudden wriggle of its abdomen (48 out of 81 mealybugs used this defense). Mealybugs also defended themselves by swiveling around the inserted stylet (37 out of 81) and, to a lesser extent, withdrawing the stylet and walking away (24 out of 81) (LM, $F = 7.56$; df = 2, 240; $P = 0.0007$). Furthermore, 24 mealybugs combined two of these behaviors and 15 used all of them. *Phenacoccus peruvianus* defended itself as a response to assessing events (antennal drumming and ovipositor tapping) and oviposition events (probing and oviposition contact) (Fig. 3). The defensive behavior of the mealybugs was influenced by their body size, as they tended to defend less as they grew larger (GLM, n = 81; $\chi^2 = 11.73$; $P = 0.0006$) (Fig. 4).

Of the 81 encounters observed, *Acerophagus* sp. successfully parasitized 42 mealybugs. Parasitism rates were influenced by mealybug defense and size. Parasitism was lower when mealybugs defended themselves (37.0 ± 6.2%), than when they did not (84.6 ± 8.9%) (GLM, n = 81; $\chi^2 = 7.81$; $P = 0.0052$). Parasitism increased in larger mealybugs (GLM, n = 81; $\chi^2 = 11.11$; $P = 0.0008$). There was no significant interaction between defense and host size (GLM, n = 81; $\chi^2 = 0.08$; $P = 0.78$) (Fig. 5).

Handling time in parasitized hosts averaged 36.45 ± 4.65 min and increased significantly when *Acerophagus* sp. parasitized larger hosts (LM, n = 42; R^2 = 9.66; *P* = 0.045) (Fig. 6). On the other hand, handling time was independent of host defense occurrence (when hosts defended: 40.70 ± 6.75 min; when host did not defend: 32.59 ± 6.44 min) (LM, n = 42; *F* = 0.0013; *P* = 0.97). There was no significant interaction between defense and host size (LM, n = 42; *F* = 2.39; *P* = 0.13).

3.3 Parasitoid residence time and behavior on ant-attended patches

The mean number of parasitoids wich remained in patches with ants for 30 minutes was significantly lower than in patches without ants (GLM, *n* = 40; χ_2= 20.55; *P* < 0.0001). Only one of the 20 released parasitoids remained for 30 minutes in a patch with ants and the overall residence time averaged 5.1 ± 1.5 minutes. On the other hand, 14 of the 20 parasitoids released on bracts without ants remained for more than 30 minutes and the overall residence time averaged 23.2 ± 2.6 minutes in the patch. The proportion of parasitoids that attempted oviposition was also influenced by the presence of ants (GLM, *n* = 40; χ_2= 4.05; *P* = 0.044). 50.0 ± 12.1 % of the parasitoids released in patches with ants attempted oviposition, but the ants disrupted the operation and constrained them to leave the patch in nine out of ten cases. On the other hand, 80.0 ± 9.2 % of the individuals released in patches with no ants attempted oviposition and all of them continued ovipositing when the observation ended.

3.4. Mealybug-ant association

216 of the 901 (28.74 ± 9.16%) mealybug colonies observed in 15 sites were tended by ants (Table 1). All the ants encountered in eastern Spain were identified as *L. grandis*. Three of the 15 sites sampled showed high levels of ant-mealybug association with more than 80% of colonies tended by ants, whereas in nine sites the presence of tending ants was sporadic with less than 20% of colonies tended by ants.

4. Discussion

Acerophagus sp. assessed its hosts by drumming their bodies with its antennae and tapping them with its ovipositor and hardly ever rejected a non-defending host , regardless of its size. This behavior during host assessment has been also recorded in other parasitoids of the same genus such as *Acerophagus mundus* Gahan*, Acerophagus flavidulus* Brèthes, *Acerophagus notativentris* Girault and *Acerophagus coccois* Smith (Hymenoptera: Encyrtidae) (Bynum, 1937; Clausen, 1924; Karamaouna and Copland, 2000; Van Driesche et al., 1987a). Among these studies, Van Driesche et al. (1987a) found similar acceptance rates for three female instars (2ndand 3rd nymphal instars, and adults) when *A. coccois* parasitized *Phenacoccus herreni* Cox and Williams. After acceptance, *Acerophagus* sp. started to probe the mealybug's body and frequently carried out circular abdominal movements. Similar movements have been closely associated with oviposition in other encyrtid species (Bugila et al., 2014b; Cadèe and van Alphen, 1997), but in our case they were only linked in 71.05% of the encounters that led to successful oviposition. Furthermore, no case of host feeding was recorded in this study. The absence of host feeding seems to be common in this genus, as has also been documented in *Acerophagus maculipennis* Mercet, *A. coccois* or *A. flavidulus* (Karamaouna and Copland, 2000; Sandanayaka et al., 2009; Van Driesche et al., 1987b). Flanders (1963) suggested that non host-feeding mealybug parasitoids are efficient natural enemies because oogenesis is less conditioned by interference by ants. Some parasitoids need to host feed to obtain nutrients for oogenesis. However, host feeding takes several minutes and lasts longer than oviposition and these parasitoids can easily be disrupted by ants (Barzman and Daane, 2001; Flanders, 1963). The absence of host feeding in *Acerophagus* parasitoids could thus contribute to their wide success as biological control agents (Bartlett, 1961; Moore 1988).

Phenacoccus peruvianus responded to parasitoid attacks with three non-excluding active defensive behaviors: wriggling, swiveling around the inserted stylet and withdrawing the stylet and running away. These defensive behaviors had previously been described in other mealybug species (Boavida et al., 1995; Bynum, 1937; Pijls, 1995) and more recently in *P. peruvianus* when attacked by *Anagyrus* sp. nr. *pseudococci* (Girault) (Bugila et al., 2014a). However, other common active

defensive behaviors in mealybugs such as secreting defensive compounds were not observed (Bokonon-Ganta, 1995; Cadèe and van Alphen, 1997; Pijls, 1995). This result differed from the study by Bugila et al. (2014), who recorded reflex bleeding in *P. peruvianus* when it was attacked by the polyphagous parasitoid *A.* sp. nr. *pseudococci*. Defensive behavior occurred as a response to ovipositor contact when the parasitoid examined the host body and oviposited, and in only a few cases was due to antennae drumming. This defense allowed the mealybugs to avoid being parasitized, and to a higher degree in larger hosts, in part as a result of their lower tendency to defend themselves. These findings do not support previous research. Cadèe and van Alphen (1997) found that larger *P. citri* defended themselves more than the smaller ones when parasitized by *L. abnormis.* The same pattern was found in *Rastrococcus invadens* Williams when parasitized by *A. mangicola* (Bokonon-Ganta et al., 1995). In a previous work, *Acerophagus* sp. mostly parasitized the oldest and largest hosts when it could choose among different mealybug instars (Beltrà et al., 2013c). The active defensive behavior of *P. peruvianus* described in this paper may thus explain the different degrees of parasitism among host instars.

Acerophagus sp. took more than 30 minutes to parasitize *P. peruvianus*. Previous observations in other species of the same genus described shorter oviposition times, ranging from 2 to 15 minutes in *A. mundus*, 5 to 20 minutes in *A. maculipennis* and 15 minutes in *A. coccois* (Bynum, 1937; Dorn et al., 2001; Sandanayaka et al., 2009). Moreover, the handling time of *Acerophagus* sp. was much longer than that spent by other mealybug parasitoids, such as *Gyranusoidea tebygi* Noyes*, Coccidoxenoides perminutus* Girault, *Anagyrus mangicola* Noyes*, Anagyrus pseudococci* Girault, *A.* sp. nr. *pseudococci* or *Leptomastidea abnormis* Girault, which only needed a few minutes to successfully parasitize their hosts (Boavida et al., 1995; Bokonon-Ganta et al., 1995; Bugila et al., 2014b; Cadèe and van Alphen, 1997; Joyce et al., 2001; Hcidari and Jahan, 2000; Zinna, 1959) (Table 2). Handling time by *Acerophagus* sp. was not influenced by host defense, but increased with mealybug length. In other studies, the secretion of defensive exudates was found to increase the time spent by the

parasitoid in grooming (Boavida et al., 1995; Bokonon-Ganta et al., 1995). However, we did not observe this defensive behavior in *P. peruvianus* and grooming time was very short compared to the time devoted to oviposition. The long handling time of *Acerophagus* sp. may be related to its large brood size, because it spends more time when parasitizing larger mealybugs, which bear larger broods (Beltrà et al., 2013c).

Handling time is an important attribute for the reproductive success of a parasitoid (Barzman and Daane, 2001; Godfray, 1994; Wajnberg, 1989). The oviposition behavior of *Acerophagus* sp. was a time-consuming process that could involve a high risk of mortality in the presence of ants and compromise its biocontrol capacity. However, *Acerophagus* sp. is now a successful biological control agent of *P. peruvianus* in eastern Spain and has significantly reduced mealybug populations. These inconsistent results raise the question of whether the parasitoid is able to overcome ant protection. Our field observations showed that *Acerophagus* sp. remained less time in ant-tended colonies and were disrupted when ovipositing. Consequently, *Acerophagus* sp. does not seem to be adapted to searching and parasitizing in ant-tended colonies and its success as a biocontrol agent can only be explained by the low percentage of colonies of *P. peruvianus* tended by ants. Ant attendance depends on honeydew quantity and quality (Mailleux et al., 2003 ; Völkl et al., 1999). The honeydew excreted by *P. peruvianus* when it feeds on bougainvillea has a poor quality for its parasitoid, and in the same way it might be unattractive for *L. grandis* (Beltrà et al., 2013c). At this point, it is difficult to predict whether *Acerophagus* sp. can be an efficient biological control agent in other areas where aggressive ant species such as *Linepithema humile* (Mayr) (Hymenoptera: Formicidae) are widespread in urban landscapes, such as in southern California (Klotz et al., 2008; Tena et al., 2013). In fact, Daane et al. (2007) encountered low parasitism rates for another parasitoid of the genus *Acerophagus*, *A. flavidulus*, when it parasitized *Pseudococcus viburni* (Signoret) in the presence of the Argentine ant *L. humile*. Therefore, the exclusion or suppression of ants should be considered to

introduce and improve the establishment of *Acerophagus* sp. in classical biological control programs in urban landscapes in which aggressive ants are abundant.

In conclusion, the present study provides a detailed description of the oviposition behavior of the biological control agent *Acerophagus* sp. and the active defensive responses of its host *P. peruvianus* against being parasitized. *Acerophagus* sp. parasitism is compromised by the *P. peruvianus* defense and, more importantly, by the considerable length of time required for oviposition, even in the absence of host-feeding, which reduces its efficacy in ant-tended colonies. Despite this long oviposition, *Acerophagus* sp. is a successful parasitoid of *P. preuvianus* in eastern Spain because of the low number of colonies tended by ants (Beltrà et al., 2013b). However, these findings should be taken into account in classical and conservative biological control programs dealing with large numbers of aggressive ants, since *Acerophagus* sp. has not adapted its behavior to parasitizing in ant-tended patches.

Table 1 Proportion of *Phenacoccus peruvianus* colonies attended by ants. Data from 15 urban green spaces in Spain and France surveyed in June and July 2012.

Sampling site	Location	Observed colonies	Ant-tended colonies	% Ant-tended colonies	Ant species
Pila	Altea	80	22	27.50%	*Lasius grandis*
Cap Negret	Altea	80	31	38.75%	*Lasius grandis*
Freres Roustan	Antibes	102	13	12.75%	Non determined
Cassin Carref	Antibes	96	9	9.38%	Non determined
Valladolid	València	57	55	96.49%	*Lasius grandis*
Blasco Ibañez	València	27	4	14.81%	*Lasius grandis*
Reig Genovés	València	37	16	43.24%	*Lasius grandis*
Calatayud	València	48	40	83.33%	*Lasius grandis*
Guàrdia Civil	València	22	0	0.00%	*Lasius grandis*
UV campus	València	21	20	95.24%	*Lasius grandis*
UPV campus	València	24	0	0.00%	*Lasius grandis*
Vivers Park	València	4	0	0.00%	*Lasius grandis*
Lluis Vives	València	160	0	0.00%	*Lasius grandis*
Santiago Rusiñol	València	60	5	8.33%	*Lasius grandis*
Esteban Castellar	València	83	1	1.20%	*Lasius grandis*

Table 2 Oviposition handling time employed by different encyrtid species when parasitizing mealybugs.

Parasitoid species	Host species	Instar	Oviposition time	Host defense	Brood size	Reference
Acerophagus mundus	*Dysmicoccus boninsis*	-	2-15 min	Yes	Gregarious	Bynum (1937)
Acerophagus maculipennis	*Pseudococcus viburni*	-	5-15 min	-	Gregarious	Sandanayaka et al. (2009)
Acerophagus coccois	*Phenacoccus herreni*	Nymphs	15 min	-	Gregarious	Dorn et al. (2001)
Anagyrus pseudococci	*Planococcus citri*	Adults	30-37 s	Yes	Solitary	Hcidari and Jahan (2000)
Anagyrus sp. nr. *pseudococci*	*Several hosts*	Adults	2-5 min	Yes	Solitary	Bugila et al. (2014b)
Anagyrus mangicola	*Rastrococcus invadens*	Nymphs-Adults	30-75 s	Yes	Solitary	Bokonon-Ganta et al. (1995)
Gyranusoidea tebygi	*Rastrococcus invadens*	Nymphs	179-740 s	Yes	Solitary	Boavida et al. (1995)
Leptomastidea abnormis	*Planococcus citri*	Nymphs-Adults	142-341 s	Yes	Solitary	Cadèe and van Alphen (1997)

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Figure legends

Figure 1. Ethogram of *Acerophagus* sp. oviposition behavior when parasitizing *Phenacoccus peruvianus*. Numbers adjacent to arrows indicate the observed frequencies of behavioral transitions.

Figure 2. Mean time spent by *Acerophagus* sp. in each action pattern when parasitizing *Phenacoccus peruvianus*. Bars (SE) with a letter in common are not significantly different ($P > 0.05$).

Figure 3. *Phenacoccus peruvianus* defensive responses to different attacking behaviors of *Acerophagus* sp. (wriggling: wriggling; moving: swiveling around the inserted stylet; getting up: getting up and withdrawing the stylet and running away; no defense: absence of defensive response.

Figure 4. Effect of *Phenacoccus peruvianus* size (length) on its approach to defending itself in presence of *Acerophagus* sp. GLM based on binomial distribution: Defense = 1/[1 + (1/(Exp((-1.524 *

Length)+2.90)))], n = 81. Data are presented slightly displaced from their original binary positions in order to better represent sample size.

Figure 5. Influence of mealybug defense and size (length) on *Acerophagus* sp. parasitism rates. GLM based on binomial distribution: Parasitism = 1/[1 + (1/(Exp(-1.197 - (1.629*Defense) + (1.791*Length))], n=81. Data are presented slightly displaced from their original binary positions in order to better represent sample size.

Figure 6. Handling time of *Acerophagus* sp. when parasitizing different host sizes. LM based on normal distribution: Log (Handling time) = 0.39*Length + 2.67, R^2 = 9.66.

Fig. 1

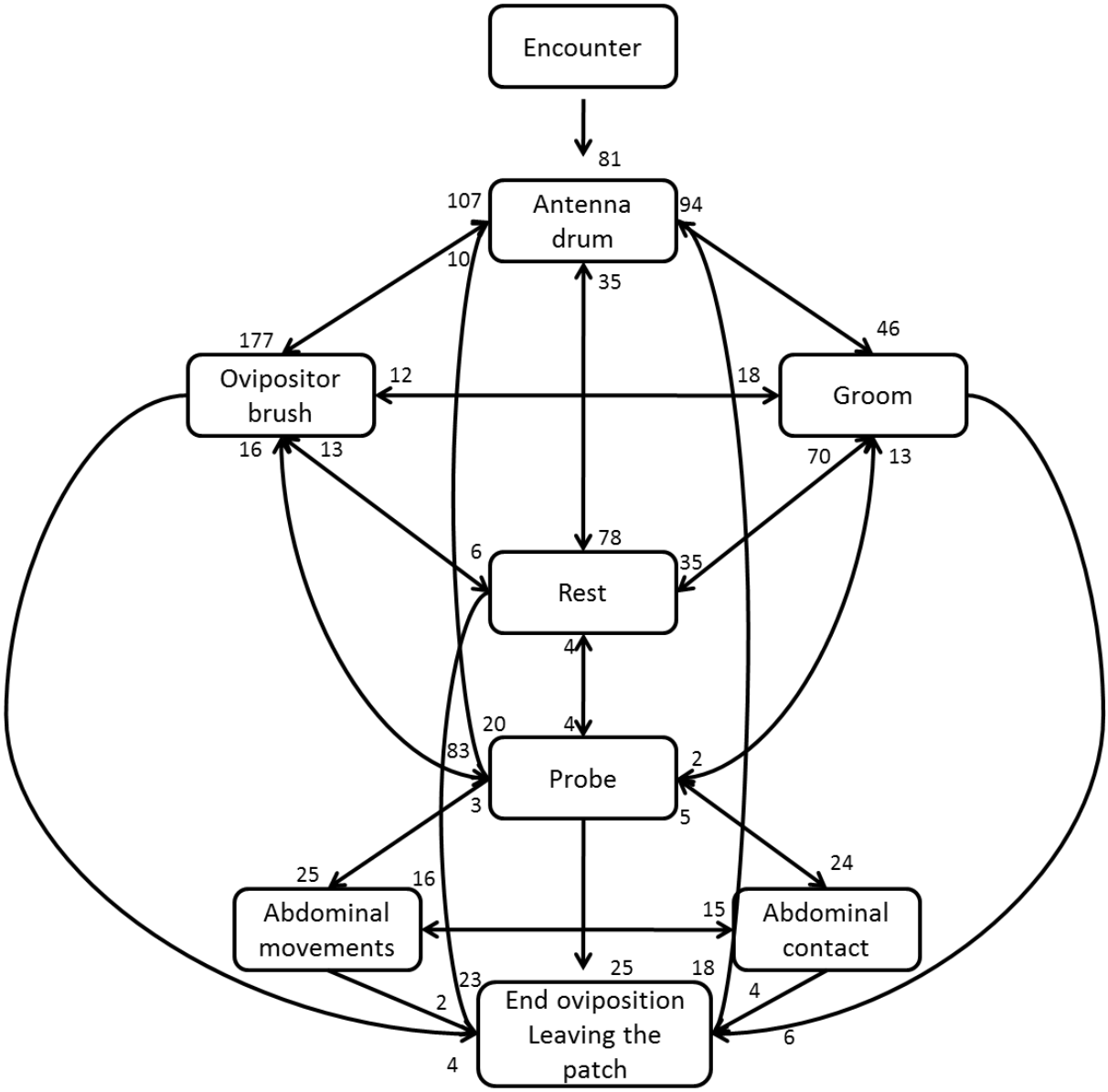
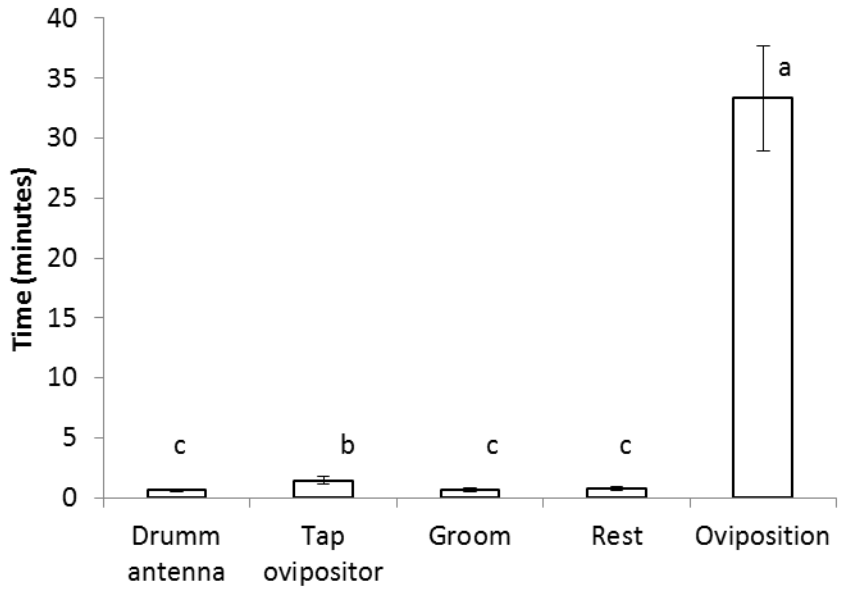

Encounter
81
Antenna drum
107
94
10
35
177
46
Ovipositor brush
12
18
Groom
16
13
70
13
78
6
35
Rest
4
20
4
83
2
Probe
3
5
25
16
24
15
Abdominal movements
Abdominal contact
25
18
23
2
4
4
6
End oviposition Leaving the patch

Fig. 2



(Antenna drumming)

Fig. 3

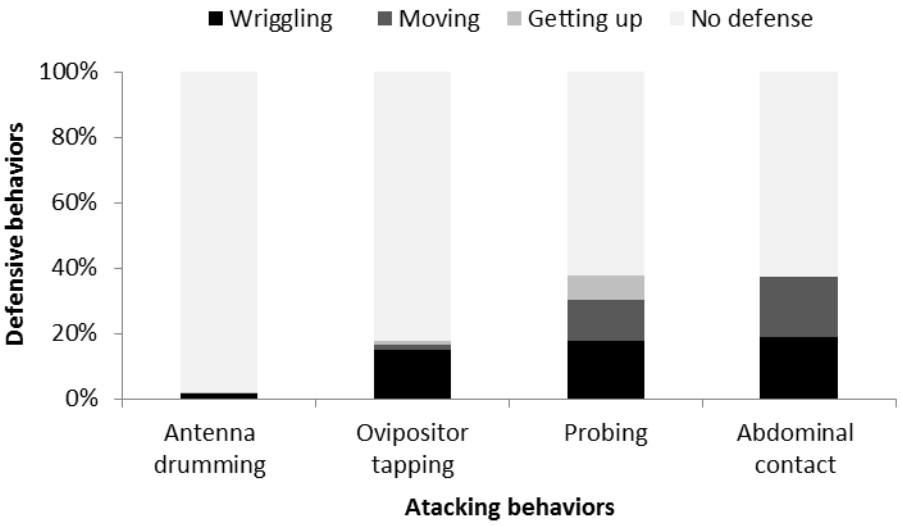

Wriggling
Moving
Getting up
No defense
100%
80%
60%
40%
20%
0%
Defensive behaviors
Antenna drumming
Ovipositor tapping
Probing
Abdominal contact
Atacking behaviors

Fig. 4

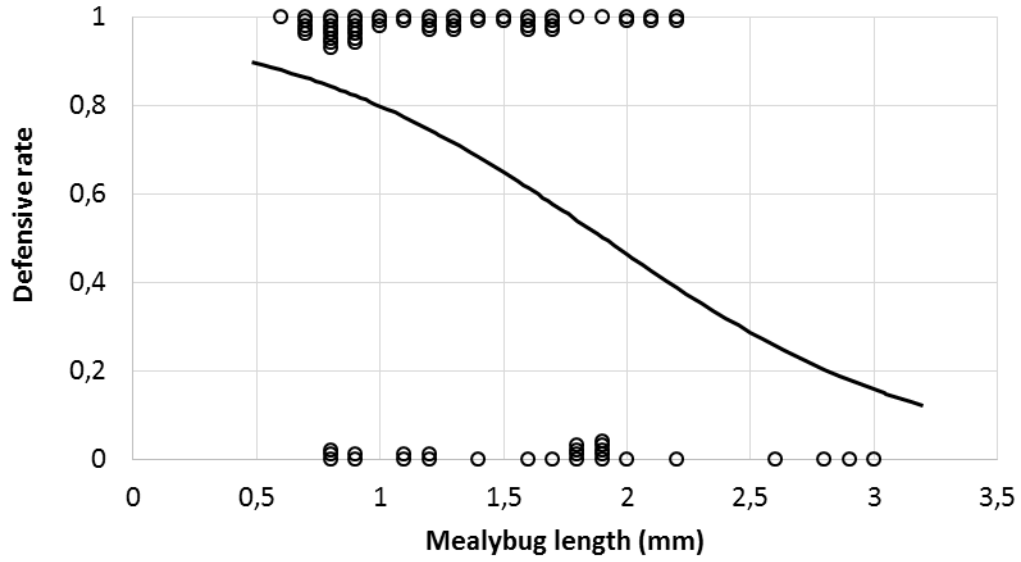

1
0,8
0,6
0,4
0,2
0
Defensive rate
0
0,5
1
1,5
2
2,5
3
3,5
Mealybug length (mm)

Fig. 5

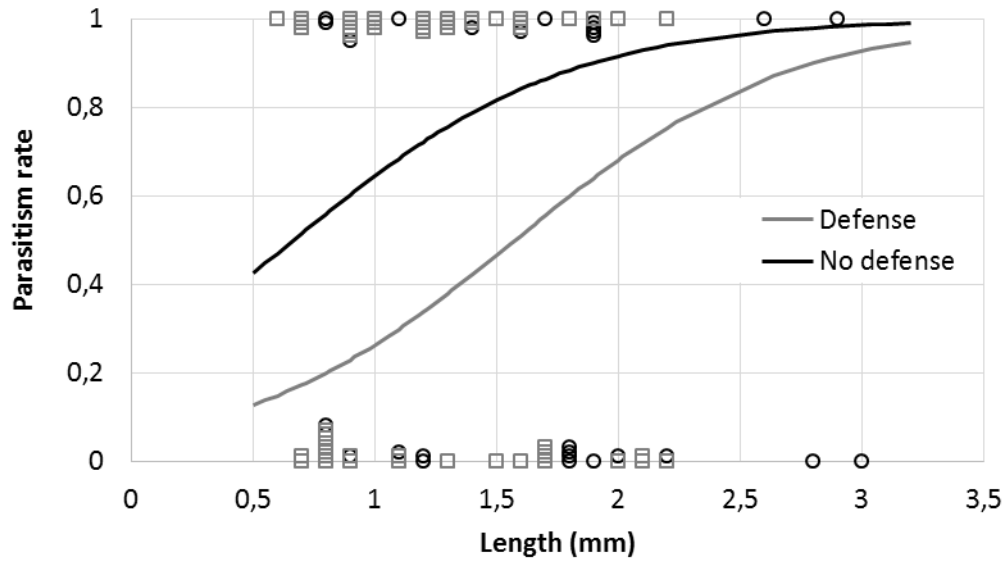
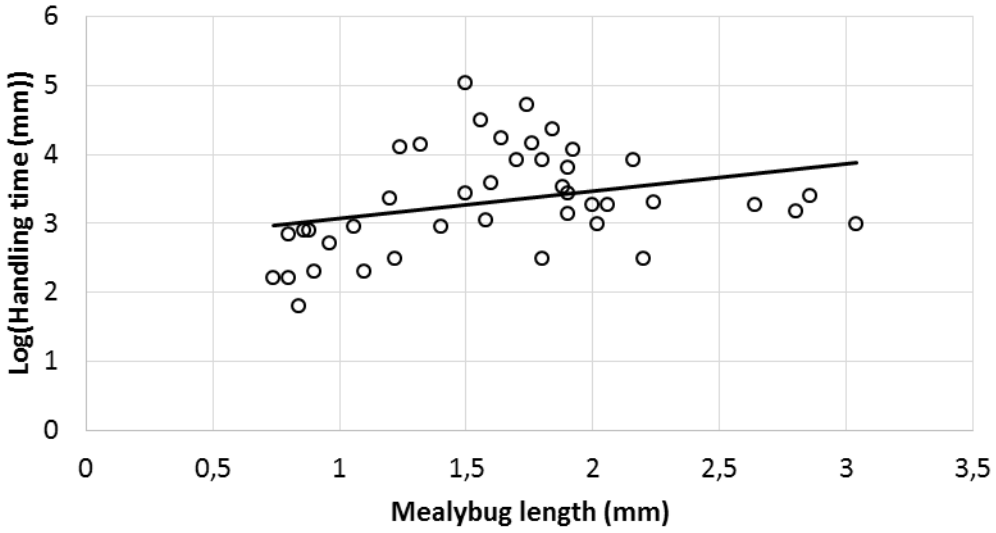

Parasitism rate
1
0,8
0,6
0,4
0,2
0
0
0,5
1
1,5
2
2,5
3
3,5
Length (mm)
Defense
No defense

Fig. 6


Log(Handling time (mm))
Mealybug length (mm)
0
1
2
3
4
5
6
0
0,5
1
1,5
2
2,5
3
3,5