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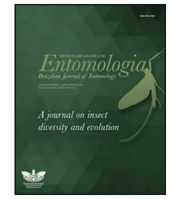

Biology, Ecology and Diversity

Biology of *Blepyrus clavicornis* (Compere) (Hymenoptera: Encyrtidae), a parasitoid of *Pseudococcus viburni* (Signoret) (Hemiptera: Pseudococcidae)

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ABSTRACT

Encyrtids (Hymenoptera: Encyrtidae) are the most important and diverse group of natural enemies of mealybugs (Hemiptera: Pseudococcidae). *Blepyrus clavicornis* (Compere) is the most common parasitoid associated with *Pseudococcus viburni* (Signoret) in the Serra Gaúcha region, Brazil. We conducted laboratory studies to assess the development time, sex ratio, adult longevity, host stage selection for parasitism, and effect of food on the longevity of adult females of *B. clavicornis*. The experiments were conducted in a climate chamber at 25 ± 1 °C, 70 ± 10% RH and 12:12 L:D photoperiod. The solitary parasitoid *B. clavicornis* parasitized third-instar and adult female stages of *P. viburni*. The development time was more than 30 days (31.75 ± 0.38 for females and 30.02 ± 0.34 for males) when *B. clavicornis* laid eggs in adult mealybug females, and 35 days (36.50 ± 0.50 for females and 34.24 ± 0.43 for males) on third-instar mealybug nymphs. The wasps did not survive longer than four days when they were fed only water, while females survived for about 30 days when fed with honey. The lifespan of females is about 20 days longer than the lifespan of males. Although *B. clavicornis* can provide significant natural control, reducing the number of individuals in the next generation by parasitizing advanced mealybug instars, we consider it unpromising for use in applied biological-control programs. Furthermore, the predominance of males in the progeny observed here suggests that *P. viburni* may not be the most suitable or preferred host for *B. clavicornis*.

Introduction

Mealybugs (Hemiptera: Pseudococcidae) are common pests in fruit production (Franco et al., 2009; Daane et al., 2012). The obscure mealybug *Pseudococcus viburni* (Signoret) is a cosmopolitan and polyphagous species found in 56 countries. It feeds on members of nearly 90 plant families and 240 genera (García et al., 2016), and is one of the main pest species of vineyards (*Vitis* spp., Vitaceae) in Brazil as well as other countries of the Americas (Correa et al., 2012; Daane et al., 2012; Pacheco da Silva et al., 2014). *P. viburni* also attacks other plants of economic importance, such as apples (Rosaceae) and pears (Rosaceae) (Ciampolini et al., 2002; Dapoto et al., 2011). It feeds on the leaves, trunks, cordons, canes, fruits and roots of host plants. Similarly to other mealybug species, *P. viburni* causes damage to leaves and grape clusters due to its copious secretion of honeydew, which provides a substrate for the development of sooty mold, responsible for losses in productivity, marketability and quality of grapes (Daane et al., 2012). Moreover, the species has been related to the transmission of *Grapevine leafroll associated virus* and *Grapevine Trichovirus* A and B, viruses that cause vineyard degeneration, and losses in the productivity and quality of clusters (Garau et al., 1995; Golino et al., 2002).

The obscure mealybug is presumed to have originated in the Neotropical Region (Charles, 2011), where a wide diversity of its natural enemies, mainly parasitoids (Hymenoptera: Encyrtidae), are present (Pacheco da Silva et al., in preparation). Biological and chemical control have been used against mealybugs, however, chemical control is often not successful due to their cryptic habit and the presence of hydrophobic waxes on the body surface (Franco et al., 2009). Hence, biological control is a good alternative method for suppressing mealybug pest populations.

The parasitoid wasp genus *Blepyrus* Howard is a little-studied group that probably originated in the New World (Noyes, 2000). Most of the 18 described species are restricted to the Americas (4 species in South America, 14 in Central America and 8 in North America); except for *Blepyrus insularis* (Cameron), which occurs

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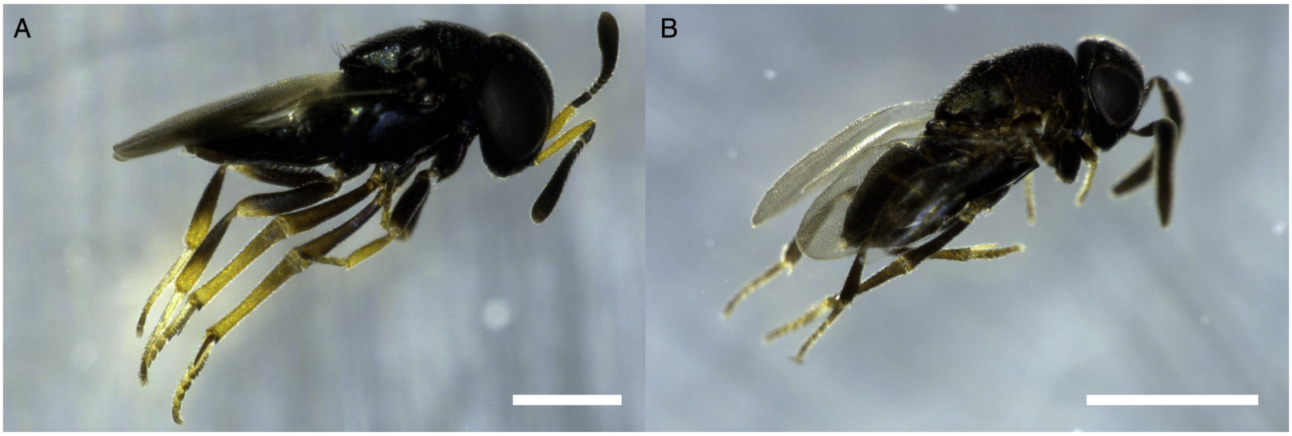

Fig. 1. (A) *Blepyrus clavicornis* female and (B) male. Scale: 1 mm.

worldwide. In southern Brazil, *Blepyrus clavicornis* (Compere), a solitary koinobiont endoparasitoid, is the commonest parasitoid developing in *P. viburni* (Pacheco da Silva et al., in preparation). Adult females of *B. clavicornis* are dark metallic blue, with part of antenna and legs yellowish and black. The body is about 2.0–3.5 mm in length (Fig. 1A) (Noyes, 2000). Males are smaller (2.0 mm long) and more hairy than females, with black bodies (Fig. 1B). Members of the genus are associated mainly with the mealybug genera *Pseudococcus* Westwood and *Dysmicoccus* Ferris (Noyes, 2016). No information about the development, reproduction and host selection of *B. clavicorns* is available.

Knowledge of the biological characteristics of natural enemies is essential in order to select potential species for use in biological-control programs. In this study we investigated certain biological characteristics of *B. clavicornis*, as a primary parasitoid of *P. viburni*.

Material and methods

Mealybug culture

A colony of *P. viburni* was established using field-collected insects from a commercial strawberry field in Farroupilha, Rio Grande do Sul (RS), Brazil (29°08.705′ S, 051°24.773′ W). Adult females were identified as *P. viburni* using the key of Williams and Granara de Willink (1992). The colony was kept in the laboratory of entomology at Embrapa Grape and Wine, Bento Gonçalves, RS. Mealybugs were maintained in sprouted potatoes *Solanum tuberosum* L. (Solanaceae), reared in plastic containers with a volume of 1 L (14 cm diameter × 9 cm deep), closed with a cover with a 10 cm-diameter aperture covered with muslin to allow ventilation and prevent the crawlers from escaping. Colonies were kept in a climate chamber at 25 ± 1 °C, 70 ± 10% RH and in total darkness.

Parasitoid culture

The rearing colony of *B. clavicornis* was established in the laboratory from adults emerged from mummified *P. viburni* individuals collected in the same commercial area where the mealybugs were collected during 2014 and 2015. The species was identified by Dr. Daniel Alejandro Aquino (National University of La Plata, La Plata, Argentina). Parasitoids were reared in acrylic cages (15 cm × 20 cm × 24 cm) with four 5 cm-diameter apertures covered with muslin to allow ventilation, and one 15-cm aperture with a sleeve consisting of a muslin tube, on *P. viburni* reared on sprouted potatoes. Parasitoid adults were fed weekly, with a streak of honey diluted in corn syrup (50:50) on aluminum foil. The colony was maintained under the same conditions as the mealybug colonies, except for the photoperiod for the parasitoids, which was 12:12 L:D (preliminary observations showed that there is no parasitism of *B. clavicornis* in total darkness). Colonies were kept in separate facilities to avoid parasitoid contamination. In order to obtain newly emerged adults for experiments, mummies were transferred with a fine paintbrush into individual gel capsules and kept in climate chambers in the same conditions described above.

Choice and no-choice test for host selection

To determine the host-stage preference of the parasitoid, the experiments were conducted in arenas consisting of a plastic container of 500 mL (11 cm diameter × 7.5 cm deep) with a cover with an 8 cm-diameter aperture closed with a muslin. Each cage contained a disk of filter paper to absorb the excess honeydew, a piece of aluminum foil (2 × 4 cm) with a streak of honey diluted in water (50:50), and a mealybug-infested sprouted potato.

Choice and no-choice tests were performed. In the choice test, 60 mealybugs, 15 from each instar (crawlers, second instar, third instar, and young adult females) were provided in the cages. To allow the mealybugs to attach to the sprouted potatoes, they were introduced into the cages 24 h before they were provided to the parasitoids. The mealybug instar was estimated according to the body length: first nymphal instar (<0.5 mm); second nymphal instar (0.8–1.0 mm); third nymphal instar (1.5–1.8 mm) and young adult females (2.8–3.2 mm) (the measurements are the mean length of at least 15 insects for each stage). Newly emerged adult couples were kept together after their emergence, and after three days they were introduced into an arena for 24 h, after which they were removed from the cage.

In the no-choice test, 20 mealybugs, all from the same instar, were provided in each cage, using the same method described above. After 24 h kept with the parasitoids, mealybugs were separated according to their stage and placed on new sprouted potatoes.

Mealybugs were observed daily and any changes were recorded. When a mummy was observed, it was removed and placed in a gel capsule until emergence. The sex ratio (number of females/(number of females + number of males), measured at emergence, and the percentage of parasitism were evaluated. At least 10 replications were carried out for both the choice test and the no-choice test. Experiments were carried out in the climate room (25 ± 1 °C, 70 ± 10% RH and 12:12 L:D photoperiod).

Determination of development time and longevity of adult wasps

For this experiment 20 young adult females and 20 third-instar nymphs fixed on sprouted potatoes were exposed to each couple of the parasitoids for 24 h. The container used in the experiment had the same conditions as described above. Newly emerged adult couples were formed, and when the females were 3 days old the couples were introduced in each cage with mealybugs for 24 h. Ten replicates (cages) were carried out for each host stage. The development time and sex ratio of the progeny were evaluated. After emergence, wasps were placed in individual glass tubes (2 cm diameter × 8 cm deep) closed with Parafilm® and fed on honey diluted with water (50:50). The parasitoids were checked daily until all individuals were dead. The development time and longevity of *B. clavicornis* was determined at 25 ± 1 °C, 70 ± 10% RH and 12:12 L:D photoperiod.

Influence of food on adult female longevity

Newly emerged adult females of *B. clavicornis* were introduced into glass tubes (2 cm diameter × 8 cm deep), closed with Parafilm®. Food was offered on aluminum foil (2 × 4 cm). The following treatments were evaluated: (i) water, (ii) honey diluted with water (50:50), or (iii) pure honey. Treatments (i) and (ii), being liquid, were offered as small droplets applied with an insulin syringe. Pure honey was offered as a streak with a 20-mL syringe. Water was supplied twice a week, and the other treatments were changed weekly. Parasitoids were checked daily until all had died. Accidental death (e.g. insects glued on honey) were excluded from the results. At least 30 replicates were carried out per treatment, kept at 25 ± 1 °C, 70 ± 10% RH and 12:12 L:D photoperiod.

Statistical analysis

The data were analyzed statistically with the software SPSS. The number of parasitized mealybugs in the choice and no-choice tests, as well as the longevity of adult females fed on different foods were analyzed with a one-way ANOVA, followed by Tukey's test at a significance level of $p < 0.05$ for mean separation. When necessary the data were transformed before analysis. The data for the proportion of parasitism between the choice and no-choice tests and the development time were evaluated using a paired *t*-test.

Results

Choice and no-choice test for host selection

The proportion of mealybugs parasitized by *B. clavicornis* differed significantly among host stages (choice test: $F = 43.75$; $df = 3$; $p < 0.001$; no-choice test: $F = 24.30$; $df = 3$; $p < 0.001$) (Fig. 2). There was no mummification of the first- and second-instar individuals. In the choice test, the number of parasitized mealybugs was significantly higher for adults (53.30 ± 7.99%) than for third-instar females (0.66 ± 0.66%). In the no-choice test, 40.00 ± 4.94% of adults were parasitized, while 23.30 ± 6.19% of third-instar females were parasitized.

Immature development time and longevity

The development time of *B. clavicornis* larvae was affected by the host stage of *P. viburni* (female: $t = -3.590$; $df = 20$; $p = 0.02$. male: $t = -7.901$; $df = 138$; $p < 0.001$), ranging from 13.75 ± 0.43 to 19.00 ± 2.00 days for females, and from 13.17 ± 0.08 to 16.43 ± 0.80 days for males, depending on the development stage of the host (Table 1).

At the beginning of the mummification, the tegument of the host mealybugs became a hard structure (mummy), considered as the parasitoid pupal stage. There was no difference in pupal development time between host stages, with a mean of 17 days of development. However, the sex of the parasitoid affected the duration of the pupal stage, when the parasitism occurred on adult female mealybugs: the parasitoid pupal duration was shorter for males and longer for females ($t = 2.238$; $df = 21$; $p = 0.03$) (Table 1).

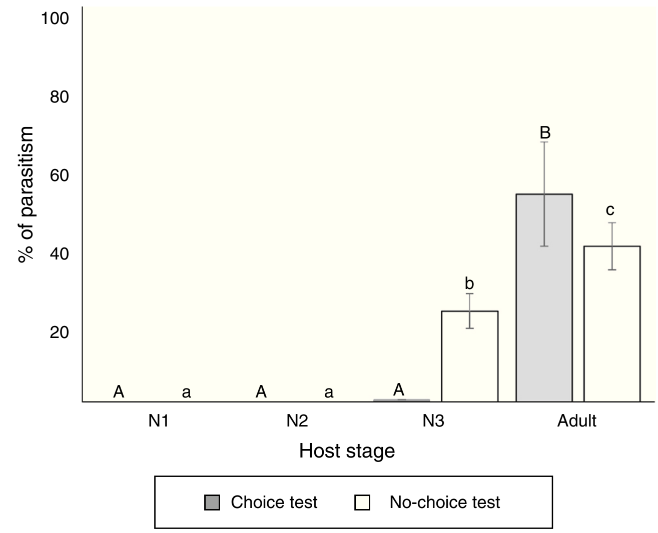

Fig. 2. Percentage of parasitism of *B. clavicornis* on *P. viburni* in choice and no-choice tests for host-instar selection. N1, first nymphal instar; N2, 2nd nymphal instar; N3, 3rd nymphal instar. Gray bars (±standard deviation) followed by the same letter (uppercase) and with bars (±standard error) followed by the same letters (lowercase) do not differ by Tukey test ($p < 0.05$).

A single parasitoid adult emerged from each mummy of the host; evidently, *B. clavicornis* is accepted as a solitary parasitoid. Survival of the parasitoid offspring (mean proportion of offspring emerged from the total number of mummies) was 58% for parasitoids that developed in adult hosts, and 66% for parasitoids that developed in third-instar hosts. For both sexes, the development time decreased with increased host age (female: $t = -3.819$; $df = 20$; $p = 0.001$, and male: $t = -7.118$; $df = 65$; $p < 0.001$) (Table 1). The development time of females was higher than males in both host stages (adult: $t = 3.339$; $df = 64$; $p = 0.02$, and third instar: $t = 3.428$; $df = 21$; $p = 0.04$).

The sex ratio of the parasitoid offspring was affected by the mealybug host stage ($t = 5.658$; $df = 8$; $p < 0.001$). Although the proportion of males was higher than females in both host instars, more female parasitoids emerged from the adult host stage (mean sex ratio = 0.307 ± 0.20), whereas the third instar produced a higher proportion of males (mean sex ratio = 0.02 ± 0.08).

The lifespan of the adult parasitoids was not affected by host instar. However, it was significantly different between the sexes (adult: $t = 2.945$; $df = 19$; $p = 0.008$, and third instar: $t = 2.238$; $df = 18$; $p = 0.01$). Female wasps lived longer than male wasps (Table 1).

Influence of food on adult female longevity

Diet significantly influenced the longevity of *B. clavicornis* ($F = 17.587$; $df = 2$; $p < 0.001$) (Fig. 3). When fed on water, the longevity was strongly affected, and neither sex survived for more than 4 days (3.90 ± 0.69 d). The longevity of the parasitoids were longer when they fed on 50% honey (33.28 ± 4.74 d), longer than those fed 100% honey (20.00 ± 2.20 d).

Discussion

The results showed that *B. clavicornis* prefers advanced stages (adult and third-instar females) of *P. viburni* for parasitism, more than younger stages (no-choice test). Nonetheless, when given a choice among different host stages, the number of parasitized mealybugs was higher on adult females. No emergence of *B.*

Table 1
Mean (±SE) development time (days) of egg to larva, pupa, and total immature stages, longevity and sex ratio of *B. clavicornis* on different host stages of *P. viburni*.

Host stage	Egg to larva		Pupa		Immature development time (egg to adult)		Longevity		Sex ratio
	♀	♂	♀	♂	♀	♂	♀	♂	
3rd Instar	19.00 ± 2.00 Aa[a]	16.43 ± 0.80 Aa	17.50 ± 2.50 Aa	17.81 ± 0.49 Aa	36.50 ± 0.50 Aa	34.24 ± 0.43 Ba	30.52 ± 3.17 Aa	11.07 ± 2.57 Ba	0.02 ± 0.08 a
Adult	13.75 ± 0.43 Ab	13.17 ± 0.08 Ab	18.00 ± 0.38 Aa	16.87 ± 0.32 Ba	31.75 ± 0.38 Ab	30.02 ± 0.34 Bb	33.28 ± 4.74 Aa	12.89 ± 0.84 Ba	0.30 ± 0.20 b

[a] Within the columns (uppercase letters), pairs of means followed by the same letters are not significantly different ($p < 0.05$), within the lines (lowercase letters), pairs of means followed by the same letters are not significantly different ($p < 0.05$).

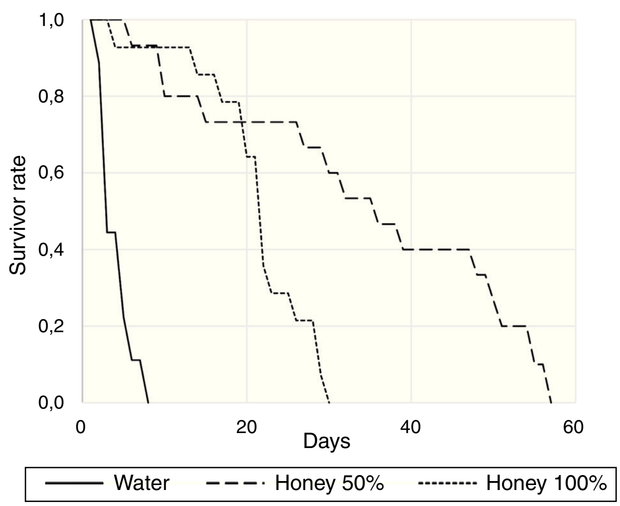

Fig. 3. Survivorship curves for *B. clavicornis*. Influence of food on longevity, for three treatments: 100% honey, honey diluted 50:50 in water, and water alone (control) (ANOVA: $F = 17.58$; $df = 2$; $p < 0.001$).

clavicornis was observed from first- and second-instar nymphs. These results corroborate the observation on *Anagyrus pseudococci* (Girault), another solitary mealybug parasitoid, similar in body size, when parasitizing *Planococcus ficus* (Signoret) (Daane et al., 2004; Güleç et al., 2006). Islam and Copland (1997) suggested some possible reasons for *A. pseudococci* preference for larger stages: (i) smaller host stages can be less frequently encountered by the parasitoids; (ii) the small body size of first and second nymphal stages can difficult the oviposition; or (iii) smaller host stages were identified by wasps as being insufficient as food resources for parasitoid development.

Another hypothesis for the unsuccessful parasitism of smaller stages could be the defenses strategies used by *P. viburni* against parasitoids. The behavioral and immune system (encapsulation) defenses have a strong impact on the parasitism rate of mealybugs (Blumberg and Van Driesche, 2001); however, even though these two strategies were not evaluated here, they were probably not the cause for the absence of parasitism in the early immature instars. The encapsulation defense of *P. viburni* against parasitoid eggs often occurs in larger-bodied hosts than in the initial, smaller stages (Karamaouna and Copland, 2009). Similarly, behavioral defense (abdominal flipping, escaping; and reflex bleeding) is a technique used by adult females against parasitoids (Bugila et al., 2014). Probably smaller mealybugs also use this technique, but not with the same efficiency as larger females. This behavior and the encapsulation of eggs and larvae could have influenced the parasitism rate of *B. clavicornis* more in larger hosts than in the first and second nymphal stages.

The complete development time of *B. clavicornis* from the parasitism to emergence of adult wasps ranges from 30 to 36 days, depending on the parasitoid sex and host stage. Mummification occurs after two weeks of parasitism, and only one wasp emerges from each mummy. The development time for parasitoids decreased with increased host age. A similar relationship between parasitoid development time and host stage was observed for other, closely related, solitary ercyrtid parasitoids, e.g. *Aenasius bambawalei* Hayat (Abdin et al., 2013) and *Aenasius vexans* Kerrich (Bertschy et al., 2007). *B. clavicornis* is a koinobiont parasitoid, and after parasitization *P. viburni* continues to grow and to lay eggs until mummification. The same was observed for *Leptomastix epona* (Walker) parasitizing *P. viburni* (Karamaouna and Copland, 2000) and for other mealybug species when parasitized by encyrtid parasitoids (Cadée and Van Alphen, 1997; Chong and Oetting, 2006). The preference for larger hosts, and the fact that the female mealybug continues to lay eggs after parasitism, can be limiting factors for the practical use of *B. clavicornis* in applied biological control of *P. viburni*.

Parasitized adult mealybugs produced more female wasps than the third-instar stage. Probably, *B. clavicornis* lay their female eggs on more suitable hosts [sex allocation, a strategy where the females choose to lay female eggs on larger hosts (King, 1993)]. Sex allocation has an important effect on biological control, since wasps that developed on larger hosts tend to have larger body sizes, which can affect the performance of the wasps as a biological-control agent (Sagarra et al., 2001). *P. viburni* females used in this study were about 3 mm long; however, in the field and in the rearing colony we observed larger pre-reproductive females, almost 4 mm long (personal observation). Apparently, under natural conditions the parasitoids have more suitable hosts available for oviposition.

The progeny observed here was male-biased in the choice and no choice-tests, where third instars produced males almost exclusively, as predicted by King (1993). A higher proportion of *L. epona* males was observed on parasitoids emerged from smaller than from larger hosts (Karamaouna and Copland, 2000). Güleç et al. (2006) also observed a male-biased *A. pseudococci* population when these wasps were parasitizing younger mealybugs. Daane et al. (2004) observed that most *A. pseudococci* females came from larger hosts. In strawberry fields in the Serra Gaúcha region, *B. clavicornis* is the most common parasitoid of *P. viburni*, and its populations are male-biased (70% of the insects collected parasitizing *P. viburni* were males) (unpublished data). Some aspects may have affected the sex population in the field, such as competition with other encyrtid mealybugs and the presence of other mealybugs in the vicinity of the strawberry field. Several parasitoid species have been collected in the same area where *B. clavicornis* was collected (Pacheco da Silva et al., in preparation). Among these wasps are species of *Anagyrus*, which studies suggest have the same host preference as *B. clavicornis* (Daane et al., 2004; Güleç et al., 2006). Other mealybugs were collected on strawberry plants in the same area, such as *Ferrisia meridionalis* Williams, but probably it was not the only species present on other plants surrounding the commercial area. *Blepyrus* species such as *B. insularis* (Cameron) and *Blepyrus saccharicola* Gahan have been reported to parasitize *Ferrisia* (Noyes, 2016). Therefore, in the field, females may have a wider choice of body sizes of *P. viburni* and other mealybug species to lay female eggs.

The lifespan of adult wasps differed between the sexes. Güleç et al. (2006) also observed that female wasps live longer than males.

Supplementary sweet food affected the lifespan of adult female wasps (Beltrà et al., 2012). Flowers can be an important source of carbohydrates for mealybug parasitoids, as observed for the encyrtid parasitoid *C. perminutus* (Davies et al., 2004). Honeydew is another source of carbohydrates used by wasps; Sandanayaka et al. (2009) observed that *Acerophagus maculipennis* (Mercet) had a similar lifespan when it fed on honey and on honeydew from *P. viburni*.

The results presented here show that adult females and third instars are the most suitable and preferred host stages for *B. clavicornis* parasitism; and when supplied with carbohydrates, female wasps can live for almost 30 days. However, the preference of *B. clavicornis* for third nymphal instars and adult females, and the fact that the mealybugs continue to lay eggs after being parasitized, are factors limiting the use of this parasitoid in biological-control programs for *P. viburni*, because releases of wasps into the field must be timed according to the availability of the preferred mealybug host instar. Furthermore, the male bias of populations observed in the field, and here confirmed in the laboratory, suggests that *P. viburni* may not be the most suitable or preferred host species for *B. clavicornis.*

Conflicts of interest

The authors declare no conflicts of interest.

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