

# Courtship, Mating Behavior, and Ovary Histology of the Nymph Parasitoid *Psyllaephagus bliteus* (Hymenoptera: Encyrtidae)

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## Abstract

The potential of the parasitoid *Psyllaephagus bliteus* Riek for the biological control of the eucalyptus pest *Glycaspis brimblecombei* Moore (Hemiptera: Aphalaridae) nymphs is high. This research sought to analyze the courtship, male competition, and mating behavior of *P. bliteus* at the proportions of 1:1 and 2:1 males to female in a Petri dish (5 cm diameter), and to describe the ovary histology of virgin and mated females of this parasitoid. At 1:1, males touch the antennae and thorax–abdomen of females during courtship, but females avoid mate attempts before they are 48 h old. At 2:1, the competition between male parasitoids inhibits mating. The histology of ovaries of virgin and mated *P. bliteus* females is similar, with two well-defined germarium and vitellarium regions, with oocytes at different developmental stages, including mature ones rich in yolk and with eggshell. A clearer understanding of the reproductive behavior and histology of *P. bliteus* aids in the use of this parasitoid for the biological control of *G. brimblecombei*.

**Key words:** biological control, mass rearing, nymph parasitoid, wing fanning

The nymphal parasitoid *Psyllaephagus bliteus* Riek is an important natural enemy of the red gum lerp psyllid, *Glycaspis brimblecombei* Moore (Hemiptera: Aphalaridae), both endemic to Australia ([Atlas of Living Australia 2020](#)).

*Psyllaephagus bliteus* was accidentally introduced with *G. brimblecombei* in Mogi-Guaçu region, São Paulo state, Brazil in 2003 ([Berti-Filho et al. 2003](#)). This natural enemy parasitizes all the five nymphal instars of the red gum lerp psyllid, although the third and fourth instars are the main target. The parasitoid lays one or two eggs in the ventral region of the thorax or abdomen per nymph of the pest ([Daane et al. 2005](#)), but only one parasitoid emerges from each nymph. *Psyllaephagus bliteus* modifies the physiology of *G. brimblecombei* nymphs, increasing carbohydrate and protein excretion for its self-protection. Parasitoid development begins when the *G. brimblecombei* nymph reaches the fifth instar ([Sullivan et al. 2006](#)).

The parasitized nymphs of *G. brimblecombei*, ‘mummies’, are dilated with opaque colors, and the parasitoid larvae consume the internal organs in the abdominal cavity of the host ([Plascencia-Gonzalez et al. 2005](#)) with a development period of 12.6 d at 26°C ([Daane et al. 2005](#)). *Psyllaephagus bliteus* pupae are exarate and

black and remain inside the host nymphs. The sharp or rounded genitalia of the females and males, respectively, allow to distinguish the sex of the parasitoid ([Riek 1962](#)). The parasitoid adult opens a hole at the posterior part of the *G. brimblecombei* nymph abdomen with its mandibles to emerge ([Plascencia-Gonzalez et al. 2005](#)).

*Psyllaephagus bliteus* controlled about 64% of *G. brimblecombei* nymphs ([Margiotta et al. 2017](#)), which is the most important strategy for the biological control of the pest in eucalyptus plantations worldwide. Environmental factors affect the populations and parasitism rates of *P. bliteus*, with values ranging from 0.21 to 7.03% in Brazil ([Ferreira-Filho et al. 2015](#)), 1.67 to 30.00% in California ([Daane et al. 2012](#)), and less than 15.00% in Italy ([Caleca et al. 2018](#)) and Portugal ([Boavida et al. 2016](#)). The longevity of adult parasitoids was negatively correlated with the temperature, from 15 to 5 d at field conditions ([Daane et al. 2012](#)). Its females lived longer than males under laboratory conditions ([Daane et al. 2005](#); A. L. Favoreto, personal information). Mass releases of *P. bliteus* increase the parasitism rate on *G. brimblecombei* in the field ([Ferreira-Filho et al. 2015](#), [Tuller et al. 2017](#)) and reduce the yield losses by this insect ([Paine et al. 2015](#)).

The efficient use of *P. bliteus* to manage *G. brimblecombei* requires knowledge on its reproduction, including the courtship and mating behavior ritual as the most important tasks (Cade 1985). The mating success varies with many factors, such as age, male competition, longevity, size (Joyce et al. 2009, Avila et al. 2016), maturation, and fecundity to mass produce the parasitoid in the laboratory.

The aim of this study was to describe, in laboratory conditions, the courtship, mating, male competition, and postmating behaviors of couples, as well as the ovary histology of virgin and mated *P. bliteus* females.

## Materials and Methods

### *Glycaspis brimblecombei* and *Psyllaephagus bliteus* Rearing

The experiments were conducted at the Laboratory of Biological Control of Forest Pests (LCBPF) in São Paulo State University (UNESP), School of Agricultural Sciences in Botucatu, São Paulo state, Brazil. *Glycaspis brimblecombei* was caged (40 cm × 45 cm × 80 cm) with *Eucalyptus camaldulensis* and/or *Eucalyptus grandis* × *E. camaldulensis* seedlings at 25 ± 2°C, 60 ± 10% RH, and 12:12-h light: dark photoperiod (Toctagon Tubular LED T8-9W, 50/60 HZ). One hundred *G. brimblecombei* adults were released per cage and, after 7 d, the eggs found on the eucalyptus seedlings were counted. These seedlings were monitored every 3 d to follow *G. brimblecombei* nymph development until the adult stage (Firmino-Winckler et al. 2009, Wilcken et al. 2010).

*Psyllaephagus bliteus* adults were obtained from mummies of *G. brimblecombei* collected in *E. camaldulensis* plantation at UNESP Teaching, Research and Extension Farm. These adults were collected using a manual insect aspirator, sexed (Riek 1962), fed on bee honey drops, and reared with *G. brimblecombei* under the aforementioned conditions. Ten to 15 pairs of *P. bliteus* were released into each cage when 50% of *G. brimblecombei* nymphs reached the third instar. After 15 d, nymphs of *G. brimblecombei* were verified and its parasitized reserved, parasitoid pupae individualized in Petri dishes (3 cm diameter) until adult emergence (Wilcken et al. 2010), which were individually placed and fed ad libitum with bee honey inside similar Petri dishes.

### Description of *Psyllaephagus bliteus* Courtship and Mating Sequence

*Psyllaephagus bliteus* courtship and mating behavior were analyzed through direct observation under stereoscopic microscope ZEISS, Leica Application Suite (version 2.1.0) software. Adults parasitoids were sexed after emergence (Riek 1962) and fed with honey bee. After this, the behavior of 50 unmated couples (1 male: 1 female) of *P. bliteus* with different ages and 20 combinations (2 males: 1 female) were analyzed. Then at 1:1, fifty couples were formed to 10 couples of newly-emerged parasitoids, 10 of 24 h-old females and newly-emerged males, 10 of newly-emerged females and 24 h old males, 10 of 48 h old females and newly-emerged and males, and 10 of newly-emerged females and 48 h old males. At 2:1, all 20 combinations of parasitoids were newly emerged. Pairs of parasitoids were formed and analyzed daily for 15 d, without replacing the dead males or females (Table 1). The experiments were carried out at 25 ± 2°C with one pair or combination per Petri dish (5 cm diameter) for 20 min. Males and females were individualized until the moment of the test and after the analysis period. The behavioral sequences (identification, courtship, mating and postmating) associate with 474 observations in the laboratory, and successful and unsuccessful mating attempts were analyzed every day until one died, as well the

**Table 1.** Longevity of *Psyllaephagus bliteus* (Hymenoptera: Encyrtidae) couples in days (mean + SE)

Couples	<i>n</i>	Longevity
Newly emerged (NE)	20	14.83 ± 2.13
24-h-old female and NE male	20	13.86 ± 1.89
NE female and 24-h-old male	20	14.73 ± 1.81
48-h-old female and NE male	20	13.74 ± 1.57
NE female and 48-h-old male	20	14.49 ± 1.46

period for the male and female to find each other, number of matings (duration), and postmating behaviors were recorded.

Preliminary assays did not indicate variations in parasitoid behavior and mating activity in the morning, afternoon, twilight, or night during observations for 60, 40, 30, or 20 min. The male parasitoid was not more active at any period, to find the females and to try to mate.

### Ovary Histology

Ten virgin females of *P. bliteus* younger than 24 h and 10-d-old mated females, kept together with males, were transferred to Carnoy's fixative (chloroform: ethanol: acetic acid, 6: 3: 1) and were then sent to the Cell Ultrastructure Laboratory of the Federal University of Viçosa in Viçosa, Minas Gerais state, Brazil. These females were dehydrated in a graded ethanol series (70, 80, 90, and 95%) for 10 min each and embedded in historesin (Leica). Samples were sectioned with 2 µm thickness in a rotative microtome, stained with hematoxylin (12 min) and eosin (30 s), and analyzed with a light microscope Olympus BX-60.

### Statistical Analyses

Before the analysis, data were submitted to normality test. The statistical analysis were performed using Kruskal–Wallis, *t*-test (significant *P*-value < 0.05) for data period time for male to find the female, and Mann–Whitney *U* statistic (significant *P*-value < 0.05) to compare the frequency of male behavior between unmated and mated couples and those of mating to the nonparametric since its dataset was not normally distributed. Data were analysed using Sigma Plot v. 11.0. for Windows software.

## Results

The conditional behavioral sequences were divided into the following phases: identification, courtship, mating, and postmating in ethogram (Table 2; Fig. 1).

*Psyllaephagus bliteus* males were active during courtship, whereas females remained passive with low response movements. Male parasitoid wasps that were released into Petri dish arena ran around the arena (latency stage) to recognize the area to begun courtship just after finding the female, whereas females stood still or, sometimes, also surveyed the arena. The male found the female in 14 s ( $H = 1.322$ ;  $P = 0.858$ ), regardless of its age (Table 3), approached, paired in front of it, touched it with its antennae, and, in some cases, intertwined its antennae with that of the female with a slight back and forth movement. Afterwards, the frequency of male antennae tapping the female thorax and abdomen, followed by chasing as a mating attempt, did not differ between mated or unmated males (Mann–Whitney  $U = 28.50$ ,  $P = 0.318$ ; Table 4). Nonreceptive females repelled males, walking off or flying away. These events were the same for newly emerged, >24 h old or for pairs with different ages. Males released into the arena immediately started courtship, repeating the whole process—antennae

**Table 2.** Sexual behavior of *Psyllaephagus bliteus* (Hymenoptera: Encyrtidae)

Behavioral acts	Description
Recognition	Males recognize females and move toward it
Antennal touch	Male antennae contacting with female antennae
Antennal intertwined	During the antennal touch, male intertwined female antennae, and male moves female with a slight back and forth movement.
Antennal drum	Male antennae tapping female thorax and/or abdomen
Chasing	Male running behind female to attempt mate
Reject	Behavioral act characterized by reject-partner during follow, females fly and/or go away from male.
Aggressive display 1	Males touch intense antennae–antennae
Aggressive display 2	Spread wings faster when the males confront
Wing fanning	Females aged 48 h old or older during receptive wing fanning communication
Abdomen movements	Females aged 48 h old or older during receptive gentle abdomen movement communication
Mount	After the behavioral act of female communication, male prepares to assuming posterior position or aligned his body on the opposite side from her.
Mate	Female stayed at stopped position during mating, while male putting aedeagus into female genital orifice and rhythmically contracting body
Postmating	Male dismount of female
Run (latency)	Moving around the arena
Immobile	Staying still in arena

**Table 3.** Period (mean + SE) in seconds (s) for *Psyllaephagus bliteus* (Hymenoptera: Encyrtidae) males to find females

Couples	<i>n</i>	Time (s)	<i>P</i>
Newly emerged (NE)	95	14.99 ± 2.12A	0.0858
24-h-old female and NE male	90	14.00 ± 1.88A	
NE female and 24-h-old male	89	13.94 ± 1.89A	
48-h-old female and NE male	116	13.83 ± 1.57A	
NE female and 48-h-old male	84	13.82 ± 1.50A	

Means followed by the same letter do not differ ( $P < 0.05$ ).

**Table 4.** Frequency of male behaviors: antennae–antennae, thorax–abdomen, mate attempts, female wing fanning, and mate in seconds for unmated and mated couples

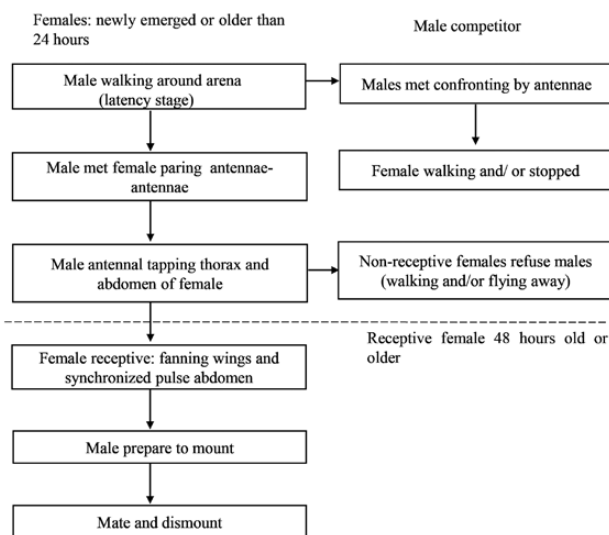
Behaviors	Unmated	Mated
Antennae–antennae <sup>a</sup>	6.20 ± 3.43 <sup>NS</sup>	4.25 ± 2.38
Thorax–abdomen <sup>a</sup>	4.90 ± 3.25 <sup>NS</sup>	3.13 ± 1.36
Mate attempts <sup>b</sup>	4.20 ± 2.44 <sup>NS</sup>	2.00 ± 0.94
Wing fanning (s) <sup>b</sup>	0.00 ± 0.00	4.25 ± 0.77
Mate (s) <sup>b</sup>	0.00 ± 0.00	15.38 ± 2.18

<sup>a</sup>Significant differences as determined by unpaired *t*-test and <sup>b</sup>Mann–Whitney *U*-test, both indicated with ( $P < 0.05$ ) nonsignificant (NS) differences.

Eight matings were observed with a mean duration of 15.4 s, varying from 5 to 32 s (5, 7, 9, 13, 15, 18, 24, and 32 s) (Table 4). Males mounted from the posterior (five of eight) or aligned under the female body (three of eight). The receptive females, 48 h old (four of eight) or older (one of 72 h old and 144 h old, one of 96 h old), fanned their wings and slowly abdomen movements. Newly emerged males initiated courtship and mated (Fig. 2B). Postmating, the parasitoids again ran or stayed still in the arena.

Males, in the 2 males: 1 female combination per arena, walked (latency stage) and, when they met each other, confronted one another with antennal movements (aggressive display 1), sometimes moving their wings (aggressive display 2) and returned to find their rival for a subsequent antennal confrontation. In this case, no courtship or mating occurred (Table 2; Fig. 1).

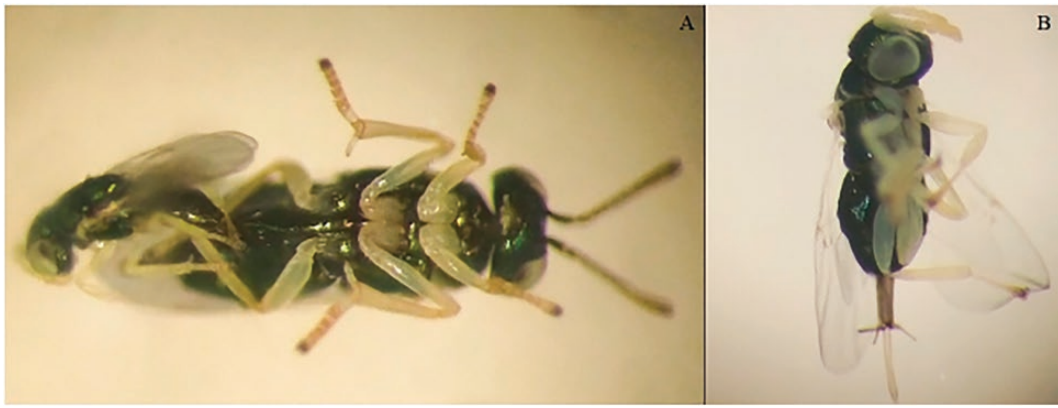
The reproductive tracts of virgin or mated *P. bliteus* females were similar (Figs. 3A–D and 4A–D). Their ovarioles were of polytrophic meroistic type with evident germarium and vitellarium regions. Large and undifferentiated oogonia and small prefollicular cells were found in the germarium. The cells undergo differentiation in the basal portion of the germarium with the nurse chambers larger than the oocytic ones, both surrounded by a single layer of follicular cells (Figs. 3A and 4A). As the oocytes moved to the vitellarium region, they increased in size and were surrounded by columnar follicular cells, resulting in an ovocytic chamber larger than the nurse ones (Figs. 3B and 4C). A total degeneration of nurse and follicular cells with a flattened aspect followed the chorion synthesis in mature oocytes (Figs. 3B, 3C, and 4D).

**Fig. 1.** Courtship and mating behavior sequence of *Psyllaephagus bliteus* (Hymenoptera: Encyrtidae).

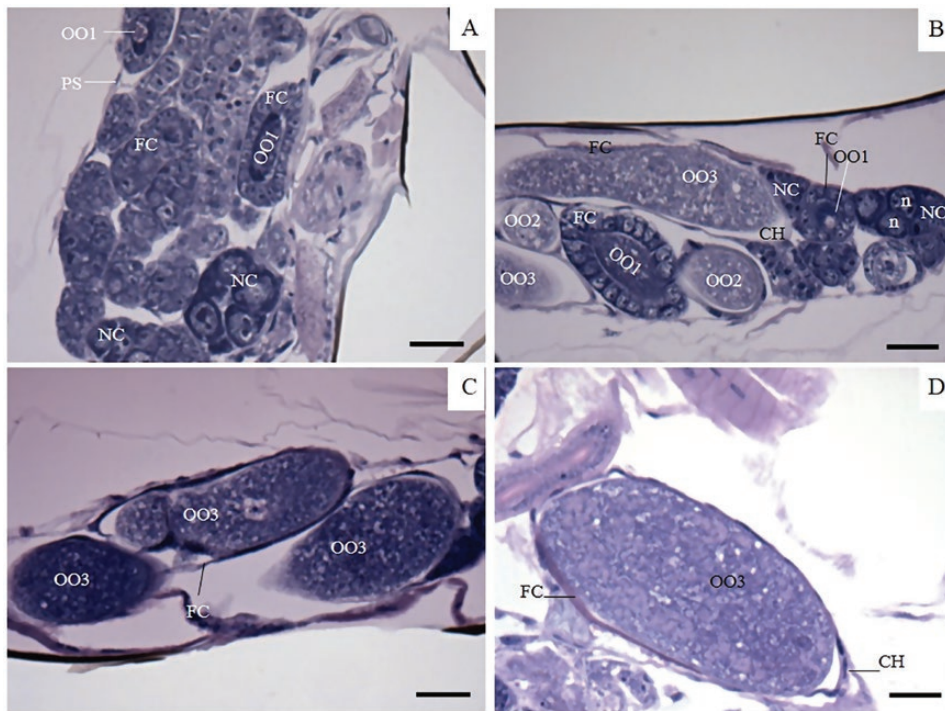
drum the female antennae ( $t(18) = 1.365$ ,  $P = 0.191$ ; Table 3) and thorax and abdomen ( $t(18) = 1.442$ ,  $P = 0.169$ ; Table 3). Females, younger than 48 h old or older, rejected male courtship, but some of them, 48 h old or older, were receptive, fanning their wings and abdomen (Mann–Whitney  $U = 0.00$ ,  $P < 0.001$ ; Table 4). In this case, the male prepared to mount or aligned its body on the opposite side, below the female body (Mann–Whitney  $U = 0.00$ ,  $P < 0.001$ ) (Table 4; Fig. 2A).

## Discussion

The male *P. bliteus* started courtship after recognizing the female with antennal tapping on female antennae and/or thorax–abdomen, a behavior crucial for Encyrtidae parasitoids, such as *Leptomastidea abnormis* Girault and *Anagyrus pseudococci* Girault (Hymenoptera: Encyrtidae) (Romano et al. 2016, 2018). Courtship behavior can stimulate female receptivity, as suggested for *Ibalia japonica* Matsumura (Hymenoptera: Ibalidae) and



**Fig. 2.** *Psyllaephagus bliteus* (Hymenoptera: Encyrtidae) mating (A) and aedeagus of newly emerged male (B).

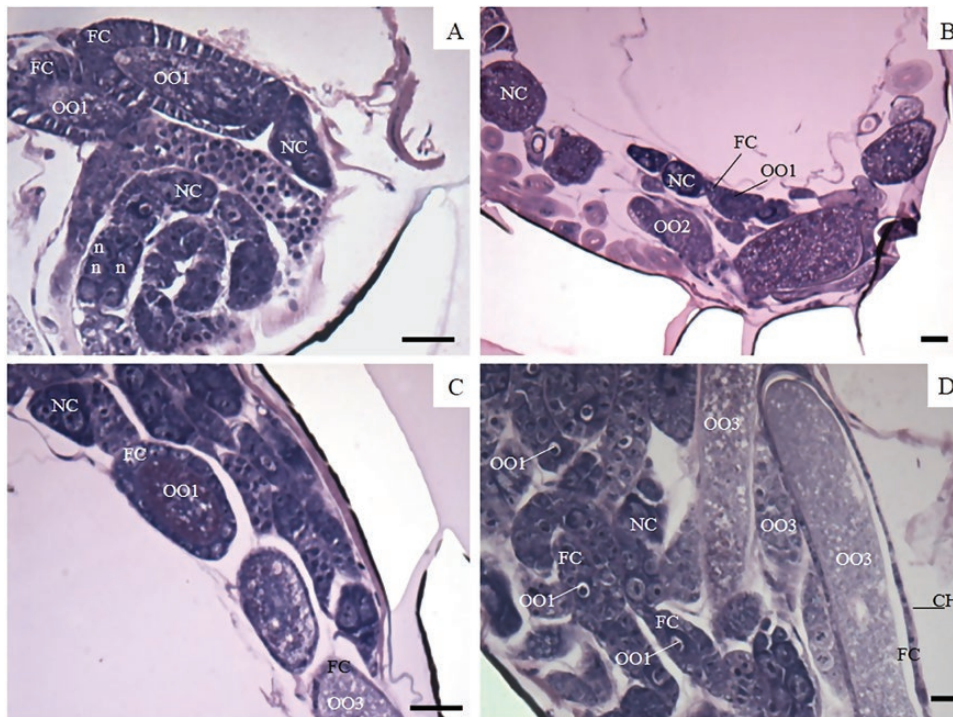


**Fig. 3.** *Psyllaephagus bliteus* (Hymenoptera: Encyrtidae) ovary of mated 240-h-old female. (A) Developing oocyte (OO1), columnar follicular cells (FCs), nurse chamber with nurse cells (NCs), and peritoneal sheath (PS). (B) Mature oocytes (OO3) with chorion (CH), oocytes in development (OO2) with flattened FCs, young oocytes (OO1), columnar FCs, and NCs. n = well-developed nucleus of nurse cells. (C) Mature oocytes (OO3); (D) oocyte detail (OO3) rich in yolk with flattened FCs and CH. Scale bar 10  $\mu$ m.

*Sphex ingens* Smith (Hymenoptera: Sphecidae), reducing courtship period and increasing reproductive success (Souza et al. 2015, 2016; Kuramitsu et al. 2019). The behavior of *P. bliteus* males intertwining females with antennae did not guarantee mating, but the females of the parasitoid *Cephalonomia tarsalis* Ashmead (Hymenoptera: Bethyridae) allowed mating after similar courtship behavior (Cheng et al. 2004). *Psyllaephagus bliteus* females, 48 h old or older, are receptive to fanning wings and abdomen movements, preparing males for mating, as described for the parasitoids *L. abnormis* and *A. pseudococci*. These females bend their abdomen dorsally and spread their wings, preparing to copulate (Romano et al. 2016, 2018). *Cleruchooides noackae* Lin & Huber (Hymenoptera: Mymaridae) mated shortly after emergence, without courtship or female receptivity for additional copulations

(Mutitu et al. 2013, Becchi et al. 2020). Rejection of mating attempts is a common behavior among females, wherein they walk or fly away from the male (Avila et al. 2016, Romano et al. 2016), such as that observed for *P. bliteus*. However, *S. ingens* females are more aggressive, partially opening their mandibles and tilting the body vertically (Souza et al. 2015).

The presence of the male competitor results in antennae-antennae fighting in *P. bliteus* as found for *Ceratosolen graveleyi* Grandi (Hymenoptera: Agaonidae) males with violent combat with biting on their mid or hindleg (Liu et al. 2019). *Cotesia urabae* Austin & Allen (Hymenoptera: Braconidae) males try to interrupt mating by pulling the successful male away from the female (Avila et al. 2016). The mating failure by *S. ingens* male manipulated the female exposure time and reduced the opportunity for other competing males (Souza et al. 2016).



**Fig. 4.** *Psyllaephagus bliteus* (Hymenoptera: Encyrtidae) ovary of virgin 24-h-old females. (A and B) Developing oocyte (OO1), columnar follicular cells (FCs), and nurse chamber with nurse cells (NCs) with large nuclei (n). (C) Developing oocytes (OO1), NCs, FCs, and mature oocyte (OO3). (D) Detail of oocyte (OO3) rich in yolk surrounded by flattened FCs and chorion (CH), many developing oocytes (OO1) with columnar FCs. Scale bar 10  $\mu$ m.

The duration of 5–32 s for the *P. bliteus* mating when females were receptive to males with fanning wings and abdomen movements is also found for the other parasitoids. The behavior in which the female ‘dances’, reported for *L. abnormis*, results in a 5.7-s mating duration (Romano et al. 2016). Nevertheless, mating period varies with species, with 2–50 s for *Cl. noackae* (Mutitu et al. 2013, Becchi et al. 2020) and 23.3 s for *C. tarsalis* (Cheng et al. 2004). Furthermore, *P. bliteus* males court virgin or mated females, similar to *Anastatus disparis* Ruschka (Hymenoptera: Eupelmidae) (Liu and Hao 2019, Liu et al. 2020), whereas *Co. unaba* was attracted only by virgin females (Avila et al. 2016).

The oocytes in the ovarioles of both virgin or mated *P. bliteus* females were at different maturation stages, including mature ones, characterizing this species as synovigenic, whereby newly emerged females present both young and mature oocytes (Jervis et al. 2001) similar to *Palmistichus elaeisis* Delvare & LaSalle (Hymenoptera: Eulophidae) (Andrade et al. 2012), *Therophilus javanus* Bhat & Gupta (Hymenoptera: Braconidae) (Souza et al. 2017), and *Eibesfeldtphora tonhascai* Brown (Diptera: Phoridae) (Farder-Gomes et al. 2019). The synchrony between egg maturation and *P. bliteus* longevity allows their females to oviposit in several host nymphs shortly after emergence, with a higher oviposition rate in the first 22 d of the parasitoid lifespan in the laboratory (Daane et al. 2005). However, *P. bliteus* longevity in the field is lower (Daane et al. 2012) but with a high parasitism rate immediately after its mass release and reaching more than 70% in the first 15 d and drops to 37% after 30 d (Ferreira-Filho et al. 2015). The decreased parasitism rate of *P. bliteus* has been correlated with its egg production and longevity, but it varies with arrhenotokous parthenogenesis (C. F. Wilcken, personal information), where males develop from unfertilized eggs (Godfray 1994, Heimpel and de Boer 2008). This is a disadvantage for the biological control because females are responsible for parasitism.

*Psyllaephagus bliteus* females and males are solitary parasitoids, i.e., females and male do not emerge at the same time. Mating is

a social behavior and this natural enemy copulates with various partners. Females of this parasitoid emerge with mature eggs and present parasitism and mating capacity, mainly, after they are 48 h old. Individuals of both sexes should remain together in laboratory rearing for at least 48 h to ensure that they mate.

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