

Ovarioles of *Jaliscoa hunteri* (Hymenoptera Pteromalidae), and effect of host and protein supplements on oogenesis

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Abstract

Jaliscoa hunteri (Crawford) (Hymenoptera Pteromalidae) is a generalist ectoparasitoid that has received attention because it can parasitize the pepper weevil *Anthonomus eugenii* (Cano) (Coleoptera Curculionidae). Since *J. hunteri* adults perform host feeding to get protein and to increase their reproductive potential, the objective of this work was to describe its ovarioles and to evaluate if feeding adults with three protein supplements (casein, pollen and hydrolysed protein) had any effect on them. Five treatments were considered: honey, honey and host, and mixtures of honey with casein, pollen or hydrolysed protein. The number of oocytes, eggs and egg size (quality) were evaluated in each case. For evaluation, we dissected 10 *J. hunteri* females at 0, 1, 5, 10, 15, 20, 25 and 30 day of age. *J. hunteri* ovarioles are of the polytrophic meroistic type, with one pair of ovaries and three ovarioles per ovary. The amount of oocytes in *J. hunteri* that were honey-host fed was 25% higher than any other treatment, followed by honey, honey-casein, and honey-pollen which produced 15% more oocytes than honey-hydrolysed protein. Honey-host produced 59% more eggs than treatments with no host, followed by honey-casein, which produced 38% more eggs than treatments with no host. Egg size was not affected by treatments. The highest production of oocytes and eggs occurred on days 5, 10 and 15, and honey-host resulted in the highest number of both variables. Though more experimentation is needed, results with no host suggest that casein has an effect on the reproductive biology of *J. hunteri*.

Key words: synovigenic parasitoids, nutrition, oogenesis, reproductive potential.

Introduction

Parasitoids require macronutrients (proteins, lipids and carbohydrates) to develop and reproduce (Rivero and Casas, 1999; Jervis and Ferns, 2004; Strand and Casas, 2008). The required nutrients can be acquired and stored during the larval and/or adult stage (Jervis *et al.*, 2001; Chen and Stansly, 2014; Kapranas and Tena, 2015). Synovigenic parasitoids need to feed continuously as adults for producing mature eggs throughout their life. During the adult stage these nutrients are obtained mainly through feeding on the host (haemolymph) but also from external sources such as nectar, honey and pollen, which provide energy and some proteins needed to reach the parasitoid's reproductive potential (Heimpel and Collier, 1996; Wäckers, 2004; Jervis *et al.*, 2008).

Protein is important for the reproductive biology of synovigenic species because it is related to the formation of ovarioles, oocyte load and egg availability and quality (Jervis *et al.*, 2008; Bodin *et al.*, 2009; Liu *et al.*, 2014). Several studies have shown that food supplements prolong synovigenic parasitoid life. However, single supplements are not sufficient for the oogenesis process to take place (Rojas *et al.*, 1996; Rivero and West, 2005; Visser and Ellers, 2012). A source of nutrients (supplements) plus host feeding can increase fertility (Morales-Ramos *et al.*, 1996; Casas *et al.*, 2005; Chen and Stansly, 2014).

Some food supplements based on carbohydrates, mainly honey, used in rearing parasitoids have the potential to influence reproductive success. Some examples for this are *Macrocentrus grandii* Goidanich (Hymenoptera Braconidae), *Neochrysocharis formosa* (Westwood) (Hymenoptera Eulophidae) and *Eupelmus vuilleti*

(Crawford) (Hymenoptera Eupelmidae) (Olson *et al.*, 2000; Casas *et al.*, 2005; Wang *et al.*, 2014). Less information exists on the effect that an exogenous protein source could have on synovigenic parasitoid reproductive biology, especially when not feeding on the host (Lauzière *et al.*, 2001; Chen and Stansly, 2014; Vanaclocha *et al.*, 2014). If an exogenous protein source could maintain the reproductive potential of a synovigenic parasitoid, at the same time than decreasing its host feeding, then it could potentially be used to decrease the cost of some laboratory rearing synovigenic parasitoids. *Jaliscoa hunteri* (Crawford) (Hymenoptera Pteromalidae) was used as study model to determine the effect of a protein-based food supplement on a synovigenic species. This wasp is a solitary, generalist ectoparasitoid that needs to feed on its host to produce eggs (Rodríguez-Leyva *et al.*, 2000). The objective of this work was to describe the ovarioles of *J. hunteri*, and to determine if the reproductive potential of adult females fed on different sources of protein is modified when they do not feed on its host.

Materials and methods

Insect rearing

For rearing the parasitoid the factitious host *Callosobruchus maculatus* F. (Coleoptera Bruchidae) was reared on chickpea seeds (*Cicer arietinum* L.) following the methodology described by Rodríguez-Leyva *et al.* (2002) and Vasquez *et al.* (2005) with slight modifications. This consisted of infesting 200 g of chickpea every other week with 300 adult bruchids. Half of this material

was kept for 21 days in plastic containers (0.5 L) under controlled conditions (25 ± 2 °C, 60-70% RH and 12:12 photoperiod) to obtain fourth instar larvae for the parasitoid. The rest of the material was kept under the same conditions until adults emerged to rear the factitious host.

For *J. hunteri* rearing, 80-100 adults were kept in a 3.8 L cage, and 200 g of infested chickpea (containing fourth instar bruchid larvae) were introduced. The infested chickpea was left 48 hours and then it was removed to 0.5 L plastic containers, under the environmental conditions described above, where the parasitoids developed and emerged. The parasitoid rearing was selected for three generations and only females that lay eggs within the first seven days were kept to increase the colony.

Treatments and experimental design

J. hunteri females ≤ 8 hours old were used for the assay. The treatments were different sources of protein mixed with honey for feeding the adults: 1) honey, 2) honey-host (infested chickpea for natural host feeding), 3) honey-casein (Sigma-Aldrich-22090-500G with 17% amino acids), 4) honey-pollen grains (chili pollen grains, 19.40% protein, Koppert Mexico), and 5) honey-hydrolysed protein (Captor 300 with 33% amino acids derived from protein hydrolysis). In all cases proteins were homogenized to 6.3%, considering some assays used in a diet for the related species *Jaliscoa grandis* (Burks) (Hymenoptera Pteromalidae) (Rojas *et al.*, 1996), and because there was not specific information for feeding *Jaliscoa* adults. Nevertheless, we considered Chen and Stansly (2014) and Vanaclocha *et al.* (2014) papers, and some previous observations which indicated that higher concentration of hydrolysed protein and casein were rejected by *J. hunteri* females (AJGS, unpublished data).

Each experimental arena consisted of 100 female and 200 male *J. hunteri* confined in a 3.8 L plastic cage with lateral openings (13×7 cm), covered with organdy fabric to allow ventilation. Water was provided with a moistened cotton wick. The treatments were offered *ad libitum* to the adults by placing fine lines of the honey and protein mixture (using a 10 mL syringe) on the upper inner face of each cage. In the case of honey plus host, honey alone and 200 g of infested chickpea with 21 days old *C. maculatus* larvae were offered. Honey or honey mixed with proteins, and infested chickpea were replaced every 48 hours according to each treatment. The assay was conducted in laboratory conditions at 25 ± 2 °C, 60-70% RH and 12:12 photoperiod. For dissection, ten females (each female was a repetition) were removed on day 0 (before feeding) and at 1, 5, 10, 15, 20, 25 and 30 days old. The assay lasted 30 days because oviposition began to decrease after this period (Rodríguez-Leyva *et al.*, 2000; Seal *et al.*, 2002), and because 80% of the parasitoid females had already died in the experimental unit. Because of large amount of material to be evaluated, females removed from the treatments were individually placed in a 2 mL Eppendorf tube and kept at -80 °C in an ultra-low freezer (Thermo Fisher Scientific, Model 703) until dissection. It was observed previously that the freezing process did not change ovarioles and eggs structure (AJGS, unpublished data).

Each of the 10 females from each treatment and time interval (0, 1, 5, 10, 15, 20, 25 and 30 days) were placed individually in a Petri dish in tap water. Under a stereomicroscope (10 \times), the abdomen was separated and the dorsal and ventral sclerites were removed with entomological tweezers. The ovarioles were then removed and photographed with a Carl Zeiss Photomicroscope III, adapted to a PAXcam 3 digital camera. Using these photographs, the reproductive system (ovarioles) were described and oocytes and eggs were counted. Only females from the treatment of honey-host were used for morphological description. The size (quality) of the eggs formed in the ovariole of the females from each treatment was also measured. To differentiate oocytes from eggs, an egg was considered mature if it had a chorion and no nurse cells (trophocytes), while nurse cells, as well as follicular tissue, were present in the oocytes (Andrade *et al.*, 2012; Chapman, 2013).

In each female, length (μm) and width (μm) of the two eggs closest to the oviduct of each ovary were measured; from the two parameters the geometric mean (Manikandan, 2011) was obtained as the egg quality (size) index. Eggs were measured using the analyser Image tool, version 3.0, for Windows (Wilcox *et al.*, 2002). The image of each egg was calibrated according to the scale established for each photograph. An exploratory analysis of correlation between the total number of eggs and egg size was also performed because there seems to be a positive relationship between the number of eggs produced by a female and egg size (Price, 1973; Blackburn, 1991).

Statistical analysis

Because the number of oocytes and eggs per treatment did not fit the normality assumption, both variables were transformed into ranges before their analysis, but original mean and standard errors were presented in results (tables and figures). The egg size (geometric mean) data were transformed to cubic root for fitting the normality assumption. A comparison among treatments and dates was made (two way ANOVA), and the Tukey separation of means test was used when there were statistical differences among treatments ($p \leq 0.05$). The variable total number of eggs and egg size could not be normalized by any transformation, and it was analysed with the Spearman correlation. The analyses were done using Statistix 8.1 (Analytical Software, 2003).

Results

Ovarioles in *J. hunteri*

Although the number of ovarioles varied from five to eight, the reproductive system in *J. hunteri* females in most cases (76%) consisted of a pair of ovaries and three long, parallel ovarioles in each ovary (figure 1A, 1B). The ovarioles are of a polytrophic meroistic type, and each is connected to an oviduct. The two oviducts in turn are connected to one common oviduct. There are also two accessory glands, each located laterally toward the common oviduct (figure 1B).

Two areas of maturation are distinguished in each ovariole. The germarium (figure 1B, 1C) is found in the distal

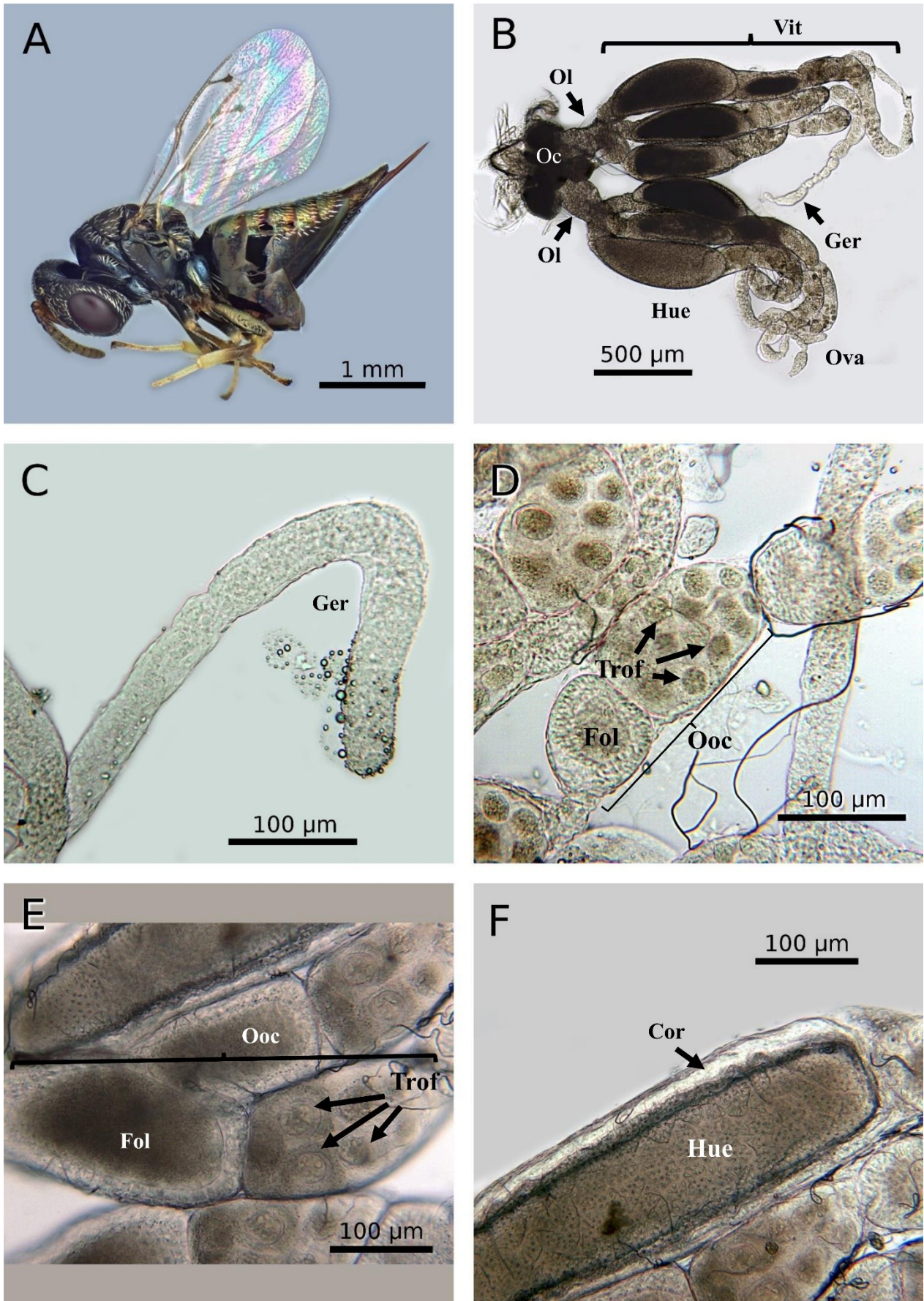


Figure 1. *J. hunteri* female (A); ovariole morphology (B); ova, ovary; Oc, common oviduct; Ol, lateral oviduct; Hue, egg (B, F); Ger, germarium (B, C); Vit, vitellarium (B); Ooc, oocytes in different stages of development (D, E); Fol, follicle (D, E); Trof, trophocytes (D, E) and Cor, chorion (F).

Table 1. Total number of oocytes and eggs (mean \pm SE) per *J. hunteri* females, and egg size (geometric mean). Females were provided with different food supplements for 30 days.

| Treatment | Variables | | |
|--------------------------|---------------------|-------------------|---------------------|
| | Oocytes | Eggs | Egg size (μ) |
| Honey-host | 263.7 \pm 12.33 a | 98 \pm 5.83 a | 310.52 \pm 3.59 a |
| Honey-casein | 195.1 \pm 8.38 b | 42.4 \pm 2.32 b | 304.66 \pm 2.46 a |
| Honey (control) | 193.8 \pm 5.49 b | 26.7 \pm 1.19 c | 314.37 \pm 2.81 a |
| Honey-pollen | 178.2 \pm 6.80 bc | 26.9 \pm 0.91 c | 310.88 \pm 2.87 a |
| Honey-hydrolysed protein | 153.3 \pm 5.55 c | 25 \pm 1.93 c | 304.64 \pm 2.88 a |

Means with the same letter in each column are not statistically different (Tukey; $P \leq 0.05$).

part and possesses germinal cells (formation of oocytes). The vitellarium occupies most of the ovariole and lines of oocytes in different development stages can be observed (figure 1B, 1C, 1E). These oocytes are characterized by a division or constriction that forms two sacks, one with follicular tissue (figure 1D, 1E) and the other with trophocytes (nurse cells) (figure 1D, 1E). As the oocytes get closer to the oviducts, they increase in size until they become eggs, which are located in the basal region, near the lateral oviducts (figure 1B). The eggs are characterized by the presence of chorion. They are hymenopteriform and lightly ornamented (figure 1F).

Oocytes, egg production and quality (size) in *J. hunteri*

There was a significant influence on the total number of oocytes because of treatments ($F_{4,49} = 24.33$; $P < 0.0001$), sampling dates ($F_{7,79} = 5.16$; $P < 0.0001$), and the interaction between the two ($F_{28,383} = 2.11$; $P = 0.0013$). Also, a significant influence on the total number of eggs among treatments ($F_{4,49} = 54.07$; $P < 0.0001$), sampling dates ($F_{7,79} = 37.39$; $P < 0.0001$), and its interaction ($F_{28,383} = 5.13$; $P < 0.0001$) was found. Females honey-host fed had the highest production of oocytes and eggs (table 1). Oocyte production in treatments honey and casein was higher than hydrolysed protein, and pollen was not different from hydrolysed protein. Females with no host that fed on honey, honey-casein and honey-pollen were able to produce 15% more oocytes than honey-hydrolysed protein (table 1). The total number of eggs produced by honey-host fed females was higher by 59.9% than honey with casein, and honey with casein was higher by 38.7% than any other treatment; egg size did not vary among treatments ($F_{4,49} = 2.5$; $P = 0.0659$) (table 1).

The honey-casein treatment had a correlation between the total number of eggs and egg size (geometric mean), more eggs but smaller. No other treatment showed a significant relationship between those variables (table 2).

Oocytes and egg dynamics in *J. hunteri* adults

Recently emerged *J. hunteri* females (≤ 8 hours) had 23.2 ± 0.8 developing oocytes, and the effect of host feeding versus protein supplements on production of oocytes was observed since day five (figure 2). After this day, females that were honey-host fed had higher numbers of oocytes on any evaluation date. On day 5, oocyte load increased in females honey-host fed relative to day 1, but after this changed the number of oocytes showed just a little variation among days 5 to 30.

Table 2. Correlation (r^2) between the total number of *J. hunteri* eggs and egg size (geometric mean) by treatment.

| Treatment | r^2 | n | P |
|--------------------------|--------|----|---------|
| Honey (control) | 0.1533 | 63 | 0.2296 |
| Honey-host | 0.0364 | 67 | 0.7695 |
| Honey-casein | 0.7036 | 60 | <0.0001 |
| Honey-pollen | 0.1671 | 61 | 0.1975 |
| Honey-hydrolysed protein | 0.2443 | 59 | 0.0624 |

Females had a similar egg load on day one in any treatment (3.96 ± 0.32), and there were no differences among them in the first two dates of evaluation (figure 3). Over time, honey-host fed females produced consistently higher numbers of eggs than those that did not have access to host. The next best treatment was honey-casein, which produced more eggs and its effect lasted practically from day 5 to 30 (figure 3).

Discussion

The morphology of *J. hunteri* ovarioles is common among hymenopteran parasitoids (Iwata, 1960; Liu *et al.*, 2014; Mao *et al.*, 2016). The number of ovarioles in this species varied from 3 to 8, but most females (76%) had only three ovarioles per ovary. This morphological characteristic is similar to *J. grandis*, a related species, and to other pteromalids such as *Trichomalopsis shirakii* Crawford and to the eupelmid *E. vuilleti* (Morales-Ramos *et al.*, 1996; Bodin *et al.*, 2009; Mao *et al.*, 2016).

The importance of nutrition and host presence in ontogenesis of synovigenic parasitoids has been well documented (Jervis *et al.*, 2001; 2008), including the related species *J. grandis* (Morales-Ramos *et al.*, 1998). In our study, differences in total number of oocytes and total number of eggs were observed, as well as differences in their development over time, a consequence of adults' access to different sources of protein supplements plus honey. In general, protein supplements had less impact on the total number of oocytes. However, this situation was very different in the total number of eggs. Honey-host was the treatment that produced the highest number of eggs (98 ± 5.8), followed by honey-casein (42.4 ± 2.3), which surpassed the rest of treatments. Because synovigenic parasitoid egg development is related to acquisition of nutrients during the adult stage (Casas *et al.*, 2005;

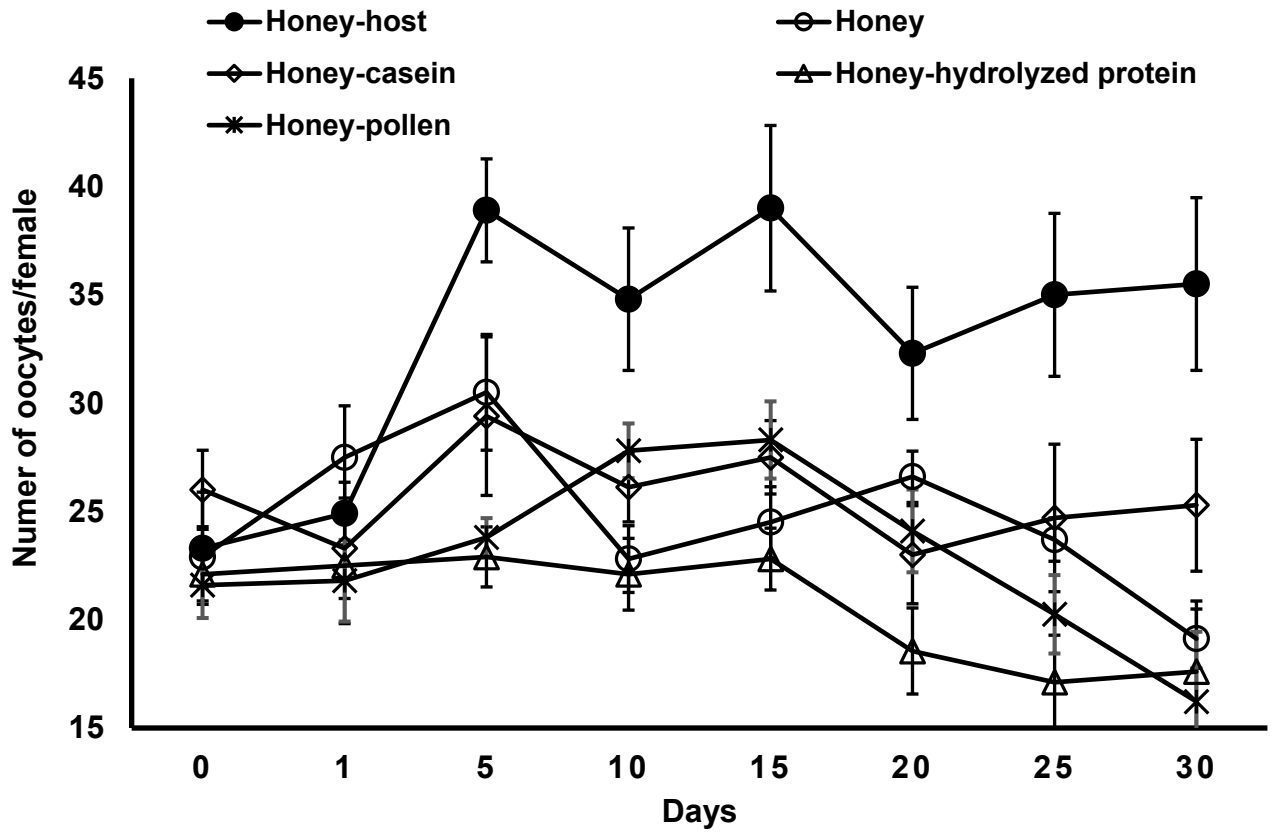


Figure 2. *J. hunteri* oocyte dynamics at different time intervals. Females were given different food supplements. Data are means and the vertical lines are standard error (SE).

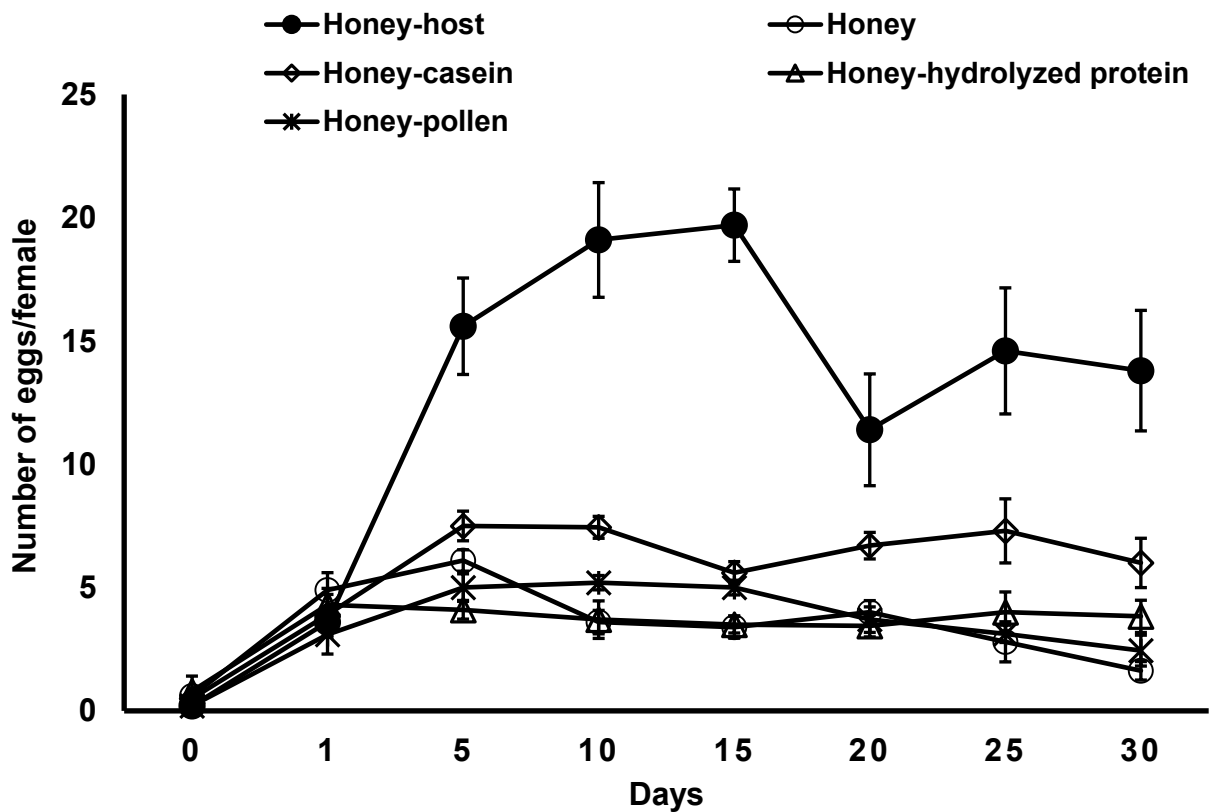


Figure 3. *J. hunteri* egg dynamics over time. Females were provided different food supplements. Data are means, and vertical lines are standard error (SE).

Jervis *et al.*, 2008; Kapranas and Tena, 2015), it is likely that honey-casein was a better source of proteins for *J. hunteri* than the rest of the treatments with no host. This differential response to protein sources in adults has been reported in species of *Aphytis* (Hymenoptera Aphelinidae) (Vanaelochia *et al.*, 2014).

The number of oocytes over time in *J. hunteri* revealed interesting facts. For example, *J. hunteri* females emerge with a number of formed oocytes, which rapidly increases with availability of host. Females with no host that fed on honey, honey-casein and honey-pollen were able to produce 15% more oocytes than honey-hydrolysed protein. In some parasitoids oocyte maturation occurs in the pupal stage using resources obtained in the larval stage, and females emerge with their oocytes load almost complete (Wang and Messing, 2003; Bodin *et al.*, 2009; Liu *et al.*, 2014). It is likely that the same occurs with *J. hunteri*, and that might help to explain why the total oocytes load and its dynamics were less affected in treatments with no host.

The number of eggs and its dynamics over time had differences. *J. hunteri* is a sinovigenic species (Rodríguez-Leyva *et al.*, 2000; Seal *et al.*, 2002), and though in our case there were no formed eggs at emergence, on day one, a small number of eggs were found (around three) in all females. So, the first eggs developed between 8 and 24 hours in any treatment even with no host. This biological aspect has also been observed in other sinovigenic species, some eggs mature during the first days after emergence but females need host feeding to produce more eggs (Wang and Messing 2003; Bodin *et al.*, 2009; Liu *et al.*, 2014). *J. hunteri* host feeding was decisive for incrementing number of eggs, as it was observed in the host-fed treatment. When there was no host, the treatment of honey-casein was advantageous for egg development and its effect lasted practically from day 5 to 30.

Egg size as proxy for egg quality is considered a biological parameter of reproductive success in parasitoids and it is related to the amount of nutrients for the individual in formation (Blackburn, 1991; Giron and Casas, 2003). In our study, the protein-based supplements with and without host did not affect *J. hunteri* egg size, as it has been observed in other studies (Giron and Casas, 2003; Bodin *et al.*, 2009). On the other hand, higher egg production has been related to smaller eggs (Blackburn, 1991; Mayhew and Blackburn, 1999). In *J. hunteri* an important relationship between the number of eggs and their size was observed in the treatment honey-casein, but not in the rest of treatments. Apparently, *J. hunteri* invested more nutrients in number (egg load) than in egg size, as has been registered in some ichneumonids, braconids, eulophids and encyrtids (Price, 1973; Kapranas and Luck, 2008).

One aspect that should be considered in sinovigenic parasitoids is that egg load and its availability depend on stimulating oviposition in presence of host; that is, the parasitoids form more eggs while they are laying eggs (Donaldson and Walter, 1988; Rivero-Lynch and Godfray, 1997). Our experimental design did not offer host feeding nor host to the supplemental protein treatments, and it is likely that *J. hunteri* females without access to host did not reach their reproductive potential because there was no stimulus from the host. Even so, casein

produced more eggs than honey, pollen, or hydrolysed protein treatments.

In summary, we corroborated that host feeding is necessary for the sinovigenic parasitoid *J. hunteri* as stimulus for oogenesis. Protein sources mixed with honey offered to female adults had little effect on oogenesis. It would, nevertheless, be convenient to experiment with some of these protein sources together with host feeding in *J. hunteri*. In this way, it may be possible to explore whether one protein source promotes relevant changes in the reproductive biology of the species.

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