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Morphological characterization of immature stages of *Habrobracon hebetor* (Hymenoptera, Braconidae) ectoparasitoid of *Ephestia kuehniella* (Lepidoptera, Pyralidae)

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Abstract

Habrobracon hebetor (Say) is a cosmopolitan idiobiont braconid which parasitizes Pyralidae larvae, a pest of stored products, such as *Ephestia kuehniella* (Zeller). The objective was to describe the morphology of immature forms of *H. hebetor* and morphological changes throughout its development. Mated females of *H. hebetor* were individualized in Petri dishes containing larvae of *E. kuehniella* for parasitism for six hours. Then, the females were removed, leaving only the eggs placed on the host. The development was evaluated every 12 hours, recording all stages and changes until the emergence of adults. Using stereoscopic optical and scanning electron microscopy, photographs of immature individuals were taken. The results showed that this parasitoid completes its development between 10–12 days. There were stages overlaps during egg to adult development. Eggs are hymenopteriform, with a smooth surface. According to cephalic capsule and larval length measurements of *H. hebetor*, it was possible to determine four instars. In general, the instars are similar to each other, differing mainly in the size and shape of segments. Larvae present a gradual loss of transparency, becoming opaquer at each successive instar. Last instar larvae distanced from the host to form the cocoon and to pupate. This study was relevant for a better understanding of the physiological interactions between *E. kuehniella* and *H. hebetor*.

Keywords
Bionomy, Pre-imaginal period, Parasitoid, Electro micrographs

Introduction

In Hymenoptera 15 superfamilies and 62 families of parasitoid are recognized (Melo et al. 2012). Braconidae is the second in number of described species (18,000) with 34 subfamilies (Quicke 2015). Braconinae, is one of the largest subfamilies, containing 2,800 valid described species distributed into more than 185 genera (Yu et al. 2012). Several species of Braconinae are potential biological control agents of Lepidoptera and Coleoptera larvae (Quicke 1997).

Habrobracon hebetor (Say) (Hymenoptera: Braconidae) is an idiobiont ectoparasitoid with a cosmopolitan distribution (Eliopoulos and Stathas 2008). It parasites mainly Pyralidae larvae, among them *Ephestia kuehniella* (Zeller) (Magro and Parra 2001, Athié and Paula 2002), considered a secondary pest of stored products because it feeds on residues left by other insects as well as processed products (Lorini et al. 2015).

The number of instars, development time and feed consumption of *H. hebetor* was evaluated by Magro et al. (2006) for the improvement of an artificial diet, however without morphological details. According to Forouzan et al. (2008) and Chen et al. (2012), the time of development of immature stages of the parasitoid *H. hebetor* decreases as the temperature increases.

The morphological characteristics of immature stages play an important role in the recognition, taxonomy and classification of parasitoid wasps (Zhao et al. 2014). For the understanding of host-parasitoid relations, the recognition of immature stages at different stages of development is relevant.

In taxonomic studies, most morphological descriptions of Braconidae are concentrated in the adult stage, while the biology and morphology of immature stages still lack information (Quicke 2015). Even though there are descriptions of the immature external morphology of some braconid species (Yu et al. 2008, Carabajal-Paladino et al. 2010, Pinheiro et al. 2010, Qureshi et al. 2016). The study by Sudheendrakumar et al. (1982) describes the biology and morphology of the immature stages of *Bracon brevicornis* (Wesmael) (Hymenoptera: Braconidae), species denominated junior synonym of *H. hebetor* (Yu et al. 2012). But there are gaps in the morphological descriptions.

Although some aspects of biology at immature stages of *H. hebetor* have been studied, researches are mainly focused on the interactions between parasitoid, host and environmental factors (Eliopoulos and Stathas 2008, Chen et al. 2012, Farag et al. 2015). The morphological characterizations of immature stages aiding in taxonomy (Gumovsky 2007). However, for this species does not well elucidated. The objective of this study was to describe the development of the immature forms of *H. hebetor* based on a detailed description of external morphology in order to provide knowledge on the recognition of the immature stages of this species.

Material and methods

Laboratory creations

Rearings of *H. hebetor* and its host *E. kuehniella* were kept in the Laboratory of Entomology of the University of Santa Cruz do Sul (UNISC), Brazil. Laboratory conditions were maintained at 28 ± 2 °C, 50 ± 20% RH and 12:12 L:D. *Ephestia kuehniella* was kept on an artificial diet consisting of wheat flour (97%) and brewer yeast (3%), following the methodology proposed by Parra et al. (2014).

Morphological characterization of immature stages and development time

Twenty mated females of *H. hebetor* were individualized in Petri dishes containing one larva of *E. kuehniella* for parasitism for six hours. Then, the females were removed and leaving just one egg placed on the host, the others removed. The development time was evaluated every 12 hours by recording all stages and changes until the emergence of adults.

For the determination of the number of larval instars, a segmented regression model was used according to Ambrosano et al. (1997), with an r^2 = 99%. Twenty individuals at each stage (egg, larva, prepupa and pupa) were evaluated. We measured the largest width and length in dorsal view and the width of the cephalic capsule using micrometric lens with a stereoscopic microscope (Motic Quimis Q764ZT). The largest width was measured from the larger portion of the eggs and from the body segments of larvae, prepupa and pupa. All stages were photographed in the same stereomicroscope previously mentioned using a digital camera (Canon EOS Rebel T3).

A sample of each stage was fixed in 25% glutaraldehyde solution in a 0.2 M phosphate buffer and distilled water for 14 days. The samples were then washed three times (30 min/wash) in 0.2 M phosphate and distilled water (1:1 ratio) dehydrated in a graduated series of acetone (30, 50, 70, 90 and 100%). They were dried (Balzers CPD030) and metallized (Balzers SCD050), after which a scanning electron microscope (Jeol JSM 6060) was performed. Electro micrographs of all immature stages were made.

To the description of each stage, one individual at every stage with an average age was used. Comments on morphological changes that may occur during each stage were added after the diagnoses. For cephalic capsule chaetotaxy, last instar larvae were used as model since the distribution of setae is the same for all larval instars and prepupa (Short, 1952).

Morphological terminologies (Fig. 1).

Abbreviations:

A abdominal segment
AS anal segment
T thorax segment

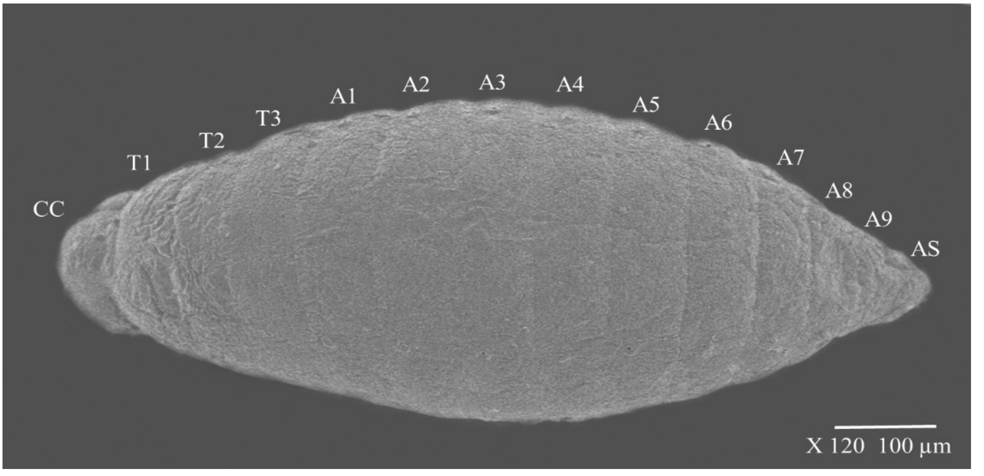

Figure 1. Second instar larva of *Habrobracon hebetor* (dorsal view) illustrating the terminology used in the text (A1-A9 abdominal segments, AS anal segment, CC cephalic capsule, T1-T3 thoracic segments).

The examined material was registered at the Entomological Collection of Santa Cruz do Sul (CESC), under lots no. 79,495 (Egg), 79,496 (Larva, 1st instar), 79,497 (Larva, 2nd instar), 79,498 (Larva, 3rd instar), 79,499 (Larva, 4th instar), 79,500 (Pre-pupa), 79,501 (female pupa) and 79,502 (male pupa).

Results

There was stage overlap (between replicates) during the egg to adult development of *H. hebetor*, indicating variations in development time. The amplitude of each immature stage was small, especially in the first instars. Thus, four days after oviposition, the larvae were already at the fourth and last instar, initiating the formation of the cocoon to pupate. The pupal phase was the longest of all development stages, lasting more than four days (Table 1).

The diagnosis of the entire development of *H. hebetor* is presented below with photographs and electro micrographs presenting some details of each stage, distribution and nomenclature of setae on the cephalic capsule, and comments on the morphological changes at each stage.

Egg

Diagnosis: opaque white, with a smooth surface (Fig. 5A), typically hymenopteriform, elongated, more or less elliptical, approximately four times longer than wide, anterior extremity (where the cephalic capsule of the embryo forms) rounded and opposite tip slightly pointed (Fig. 3A and B).

Table 1. Development time and sizes of immature stages of *Habrobracon hebetor* in *Ephestia kuehniella* larvae (12 h photophase, 28 ± 2°C and 50 ± 20% RH).

Stage/instar	Duration (Days ± SD)	Body length (mm ± SD)	Maximum body width (mm ± SD)	Cephalic capsule width (mm ± SD)
Egg	1.35 ± 0.343	0.52 ± 0.056	0.12 ± 0.011	-
Larva 1	0.73 ± 0.089	0.44 ± 0.073	0.10 ± 0.019	0.10 ± 0.014
Larva 2	0.41 ± 0.050	0.89 ± 0.142	0.36 ± 0.022	0.18 ± 0.019
Larva 3	0.90 ± 0.110	1.87 ± 0.283	0.60 ± 0.086	0.24 ± 0.019
Larva 4	1.22 ± 0.150	2.67 ± 0.139	0.90 ± 0.079	0.30 ± 0.026
Prepupa	1.97 ± 0.374	2.90 ± 0.182	0.84 ± 0.035	0.38 ± 0.022
Female pupa	4.47 ± 0.413	2.46 ± 0.113	0.91 ± 0.037	0.56 ± 0.011
Male pupa		2.50 ± 0.164	0.78 ± 0.027	0.56 ± 0.013

Measurements: overall length: 0.52 mm; maximum width: 0.12 mm.

Comments: approximately 12 hours after oviposition, it is possible to observe the embryo in formation and its development (Fig. 3B), and later the hatching.

Larva

It was possible to determine four larval instars of *H. hebetor* based on cephalic capsule length and larval body length, (Table 1) using the segmented regression model (r^2 = 99%).

In general, the four larval instars are similar to each other, differing mainly in the size and shape of segments. Larvae present a gradual loss of transparency, becoming opaquer at each successive instar with the enlargement of the intestine. Each instar had a different development time (Table 1).

First instar

Diagnosis: spherical cephalic capsule, width equal to the length of three thorax segments together, visible short antennae below the vertex region, sparse setae in the frontal region of the cephalic capsule (Fig. 2), body with 13 post-cephalic segments: three thoracic segments (T1-T3) and ten abdominal, including one anal segment (A1-A9, AS). A1-A5 had an almost equal length and width, A6-A9 width gradually decreasing up to the AS (Fig. 3C), a pair of spiracles per segment T and A (Fig. 5C), AS width equal to one third of the cephalic capsule width, smooth body surface, no setae (Fig. 5C).

Measurements: overall length: 0.42 mm; cephalic capsule: 0.10 mm; maximum width: 0.10 mm.

Comments: at the initial phase of the first instar, the larva is translucent and the cephalic capsule is as wide as the following segments. As the larva develops, its body grows rapidly and the segmentation becomes more noticeable. During the first instar, the thoracic segments exceeded the width of the cephalic capsule.

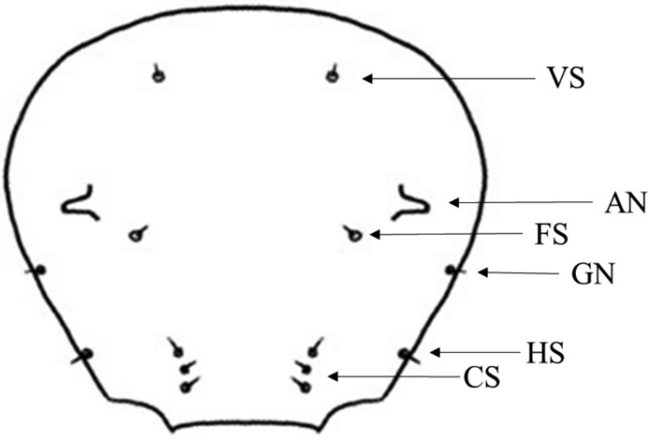

Figure 2. Cephalic capsule (frontal view) of fourth instar larva of *Habrobracon hebetor* illustrating chaetotaxy (AN antenna, CS clypeal setae, FS frons setae on the antennal region, GN genal setae, HS hypostomal setae, VS vertex setae).

Second instar

Diagnosis: spherical cephalic capsule, almost twice as wide as that of the first instar, short visible antennae with sparse setae in the frontal region of the cephalic capsule (Fig. 2), T2 and T3 twice as wide as the cephalic capsule, segments A1-A5 almost with the same length and width of T2-T3, A6-A9 with width gradually decreasing up to the AS (Fig. 3D), a pair of spiracles per segment T and A (Fig. 5C), AS with half of cephalic capsule width, smooth body surface, no setae (Fig. 5C).

Measurements: overall length: 0.92 mm; cephalic capsule: 0.18 mm; maximum width: 0.36 mm.

Comments: the width of the cephalic capsule increases, but less than the width of the body segments. With the increase in larval body size, the intestine occupies an increasing volume, reaching up to a third of the body's space at this phase of development.

Third instar

Diagnosis: spherical cephalic capsule 2.5 times larger than that of the first instar, visible short antennas with sparse setae in the frontal region of the cephalic capsule (Fig. 2), posterior part of the cephalic capsule covered by T1, T2 and T3 twice as large as the cephalic capsule, A1-A6 segments 1.5 times wider than T2-T3, A7-A9 with width gradually decreasing up to the AS (Fig. 3E), a pair of spiracles per segment T and A, width of the AS equal to two-thirds the cephalic capsule width, thoracic and abdomen dorsal with short and dense setae on all surfaces (Fig. 5B) along with sparse trichoid sensilla three times longer than setae (Fig. 5D), smooth ventral side without setae.

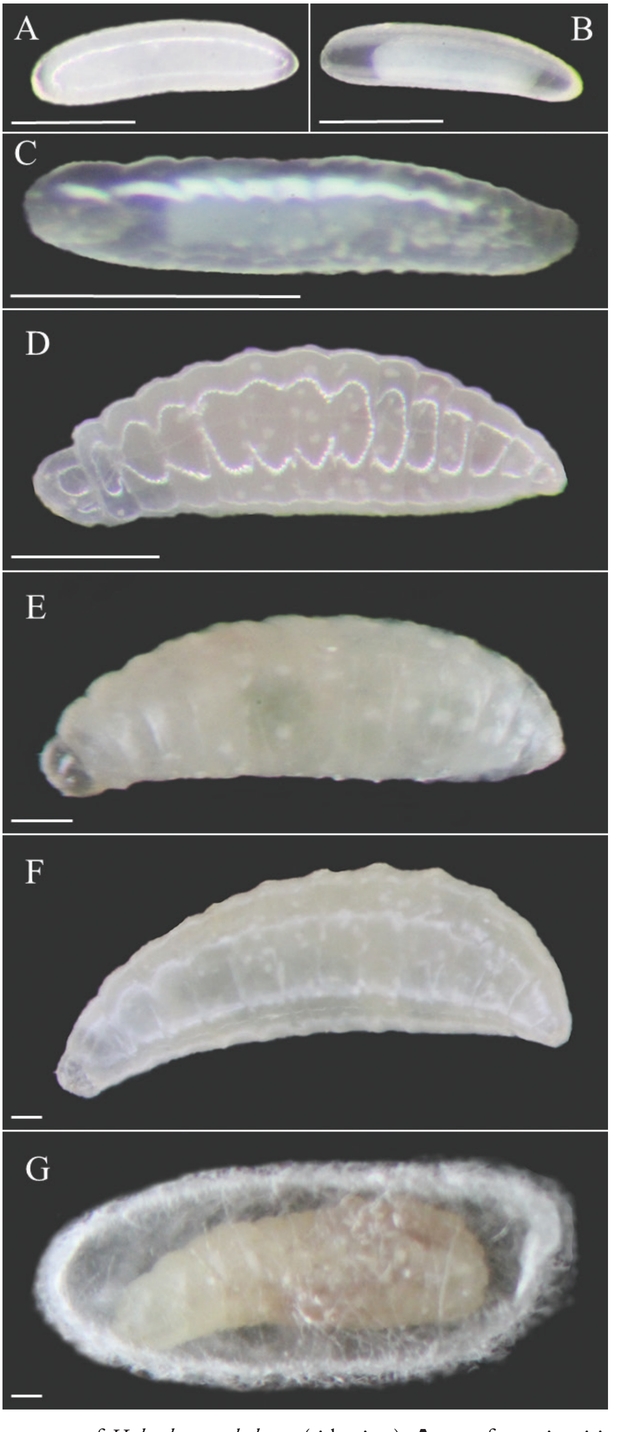

Figure 3. Immature stages of *Habrobracon hebetor* (side view): **A** egg after oviposition **B** embryo in development **C** first larval instar **D** second larval instar **E** third larval instar **F** fourth larval instar **G** cocoon in formation. Scale: (**A–C**) 0.25 mm (**D–G**) 0.5 mm.

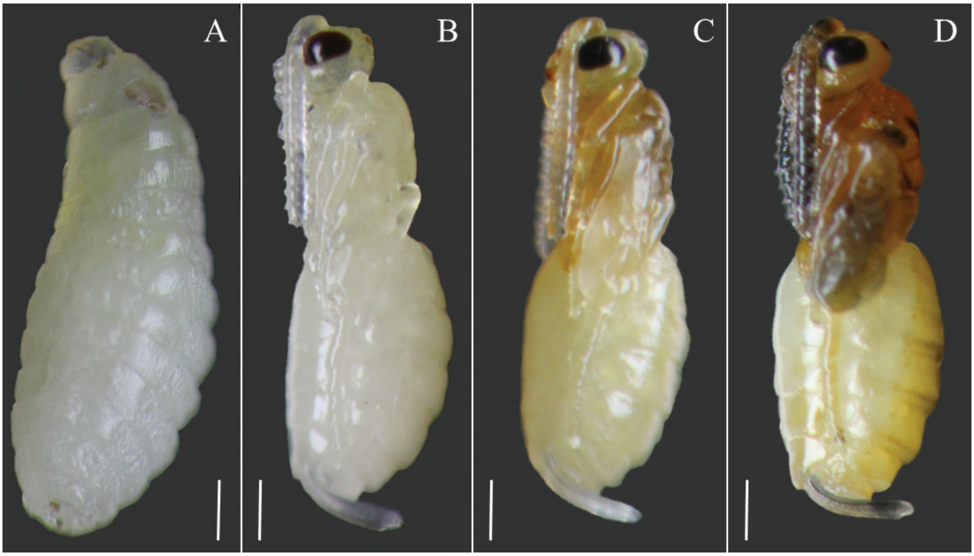

Figure 4. Immature stages of *Habrobracon hebetor* female (side view): **A** Prepupa **B** first pupal phase **C** second pupal phase **D** third pupal phase before adult emergence. Scale: 0.5 mm.

Measurements: overall length: 1.52 mm; cephalic capsule: 0.24 mm; maximum width: 0.62 mm.

Comments: as the body segments increase in size, the cephalic capsule begins to be covered in the back by the T1, reaching up to a third of the cephalic capsule. Unlike the first and second instar, the surface has short, dense setae, easily visible, scattered across the thorax and abdomen. Trichoid sensilla are developed, with a base and cone shape, reaching twice the size of setae. The larva becomes opaquer and the intestine occupies two-thirds of the body at this instar.

Fourth instar

Diagnosis: spherical cephalic capsule 3 times larger than that of the first instar, visible short antennas with sparse setae in the frontal region of the cephalic capsule (Fig. 2), cephalic capsule covered dorsally by T1, approximately with the same width, T2 twice as large as T1, T2 and T3 twice as long as the cephalic capsule, A1-A8 segments with the same length and almost four times wider than the cephalic capsule (Fig. 3F), a pair of spiracles per segment T and A, AS with the same width as the cephalic capsule, thoracic and abdomen dorsal with short and dense setae on all surfaces (Fig. 5B) along with sparse trichoid sensilla three times longer than setae (Fig. 5D), smooth ventral side without setae.

Measurements: overall length: 2.64 mm; cephalic capsule: 0.29 mm; maximum width: 0.95 mm.

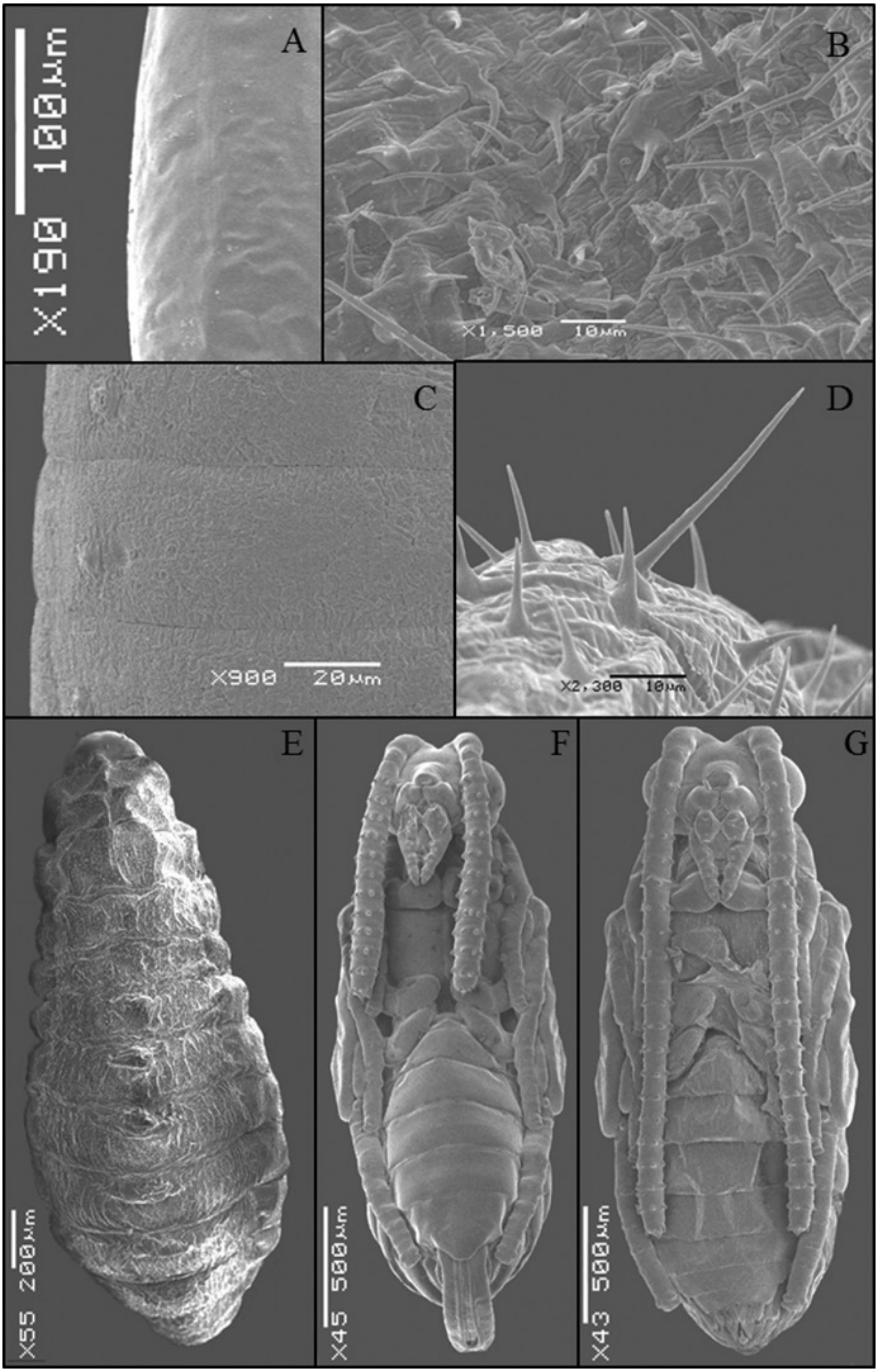

Figure 5. Scanning electron micrographs of immature stages of *Habrobracon hebetor*: **A** detail of the smooth surface of the egg **B** detail of the setae on the dorsal surface of the thorax and abdomen of third and fourth larval instars and prepupa **C** detail of the smooth dorsal surface of first and second larval instars with spiracles **D** detail of a trichoid sensillum **E** prepupa **F** female pupa **G** male pupa.

Comments: as they grow, body segments increase in size, making the cephalic capsule proportionally the smallest part of the larva in addition to being almost totally involved dorsally by the first thoracic segment. Many granules appear as small white patches scattered under the cuticle of the abdominal segments. Approximately 84 h after parasitism, larvae of the fourth instar had already consumed almost all the tissues of the host and distanced themselves from it to initiate the formation of the cocoon, which is woven with silk produced by the labial glands, forming a thick layer of threads over its body (Fig. 3G).

Prepupa

Diagnosis: cephalic capsule distinctly separated from the rest of the body, with an enlargement of the posterior lobe (Fig. 4A), visible short antennas with sparse setae in the frontal region of the cephalic capsule (Fig. 2), T1 almost as long as T2 and T3 together, T2 and T3 four times wider than long, thoracic segments separated from abdominal segments by a slight constriction, A1-A6 with length and width almost equal to T3, A7-A9 with width gradually decreasing up to the AS (Fig. 5E), a pair of spiracles per segment T and A, AS width equal to one third of the cephalic capsule width, thoracic and abdomen dorsal with short and dense setae on all surfaces (Fig. 5B), sparse trichoid sensilla three times longer than setae (Fig. 5D), and smooth ventral side without setae.

Measurements: overall length: 2.90 mm; cephalic capsule: 0.38 mm; maximum width: 0.84 mm.

Comments: at the end of the fourth instar, the prepupal transformation occurs with the fully formed, oval-shaped and white-shaped cocoon, a lighter color and absence of movement. With the connection of the midgut to the posterior intestine, there is the elimination of the meconium, which is adhered to the cocoon, making the intestine translucent. There is a differentiation in the cephalic capsule with the expansion of the posterior lobe, where, at the end of this stage, the composite eyes and the three dorsal ocelli become visible, presenting a reddish-brown coloration.

Pupa

Female diagnosis: characteristics of the head as in adults, pigmented eyes and ocelli fully formed, antennas curved down to the thorax, ending in the insertion of the last pair of legs, 13 flagellomeres of equal size, 1.5 times wider than long, containing a ring of eight spines in each segment, spines at the base as large as long with approximately half the length of each flagellomere, buccal apparatus with sclerotized mandibles, thorax as in adults but with wing structures folded laterally to the thorax, reaching A2, legs developed close to the body, abdomen with nine segments, ovipositor curved upward at the back of the abdomen (Fig. 5F).

Measurements: overall length: 2.46 mm; cephalic capsule: 0.56 mm; maximum width (abdomen): 0.94 mm.

Male diagnosis: pupa similar to that of female, differing in the longest antennas reaching ventrally A5, containing 20 equal-sized flagellomeres, as large as long, with a ring of eight spines in each segment, spines at the base as large as long with approximately one-third of the length of each flagellomere, absent ovipositor (Fig. 5G).

Measurements: overall length: 2.50 mm; cephalic capsule: 0.56 mm; maximum width (abdomen): 0.76 mm.

Comments: exarate pupa is protected by the cocoon produced by the last instar larva. Initially, only eyes and ocelli are pigmented (Fig. 4B). The mandibles and mesoscale become sclerotized, followed by the remaining parts of the thorax, acquiring an orange coloration (Fig. 4C). The abdomen, the ovipositor and the antennae are the last parts of the body to become pigmented, making the abdomen brownish yellow and the other parts brown (Fig. 4D). On average, five days after turn into pupa, the adult breaks the cocoon with its jaws and emerges, leaving the cocoon.

Discussion

The time of development from egg to adult emergence was similar to that reported for *H. hebetor* by Serra (1992), Magro et al. (2006) and Alam et al. (2014). Between 26-28°C, the immature stage occurs after 10-12 days in *E. kuehniella* larvae or other hosts. The same authors concluded that the time of development of this parasitoid is directly dependent on temperature. It can be faster at high temperatures. The development time of *B. brevicornis* evaluated by Sudheendrakumar et al. (1982) for each stage of development can not be compared with the present study. The authors do not mention the temperature at which the parasitoid was reared, since this factor is determinant to development time.

This work evaluates in more depth information about the development stages of *H. hebetor* reported in the article by Sudheendrakumar et al. (1982) by descriptions and colored photographs of all immature stages. Also scanning electromyography was used to detail structures difficult to observe under optical microscopy.

The detailed diagnoses of all immature stages are a complement to the study conducted by Alam et al. (2014), who made brief comments regarding the development time and the basic morphological characteristics of the stages of *H. hebetor* parasitizing *Galleria mellonella* L. (Lepidoptera: Pyralidae), however without associating images and a detailed diagnosis of each period. The confirmation of the morphological characteristics before emergence is important to recognize the species also at the immature stage while it is over its host.

Eggs with an elongated hymenopteriform shape were expected because, as Ide et al. (2006) reported, most Hymenoptera eggs have such a format. However, according to the same author, the corium may be smooth or rough and may present hooks. In *H. hebetor*, the egg's corium is smooth, without other structures.

The existence of four larval instars of *H. hebetor* was found by adjusting the total length and width of the cephalic capsule measurements data by a segmented regression model (Ambrosano et al. 1997) differing from the previous assessment by Magro et al. (2006), who, based on the size of jaws, recorded the existence of only three larval instars for this parasitoid using the same host. This was also opposite to the result observed by Sudheendrakumar et al. (1982), in which the authors reported the occurrence of five larval instars according to length of mandible and diameter of prothoracic spiracle. However, comparing measurements of body length, maximum body width and cephalic capsule width, it is possible to verify that the fourth and fifth instars correspond to the fourth instar described in the present study.

According to Costa and Ide (2006), the definition of the number of instars may be variable according to the methodology used for its determination, and may even be different among individuals of the same species. Thus, we reinforce the relevance of our results, considering that the morphological differences are sufficient to define the four larval instars.

Larvae with low structural complexity and successive pigmentation changes in body color had been reported for other species of parasitoids. Thomazini et al. (2000), Bittencourt and Berti Filho (2004), Yu et al. (2008) and Zhao et al. (2014) reported that the body of parasitoids becomes opaquer and less translucent throughout their development.

In the third and fourth larval instars were observed short and dense setae on all dorsal surface of the thorax and abdomen. In the description of *B. brevicornis* made by Sudheendrakumar et al. (1982) the authors reported the occurrence of these setae only in the last instar, but it was possible to observe in electronic microscopy that the setae come from the third instar.

The appearance of white granules along the abdomen at the last larval instar has also been reported for a parasitoid of the same superfamily, *Diadromus collaris* (Gravenhorst) (Hymenoptera: Ichneumonidae) (Zhao et al. 2014). According to Parra (2009), such globules are fat bodies that store lipids and supply long-term energy reserves for adults. These lipids accumulated during the larval stage provide the adults with energy for approximately 10 days (Istvan et al. 2011).

Four days after oviposition, the parasitoid is already at the last larval instar and moves away from the host. This behavior is justified because, as reported by Quicke (1997) for ectoparasitoid, there is a potential risk of putrefaction of non-consumed host tissues. As a consequence, the parasite tends to develop rapidly at the larval stage. This reduces the effects of any decline in host quality which occurs naturally or as a result of infections by microorganisms (Quicke 2015).

After leaving the host, the parasitoid begins the construction of the cocoon to pupate. The formation of the cocoon with a thick layer of silk, according to Tagawa and Kitano (1981), indicate its importance for the survival of ectoparasitoid, as it is a protection against physical damage, predators, hyperparasitoids and desiccation. An exarate pupa is formed, with appendices separated from the body, as in most Hymenoptera (Ide et al. 2006). In addition, the morphological description made by Sudheendrakumar et al. (1982) of the pupa stage for female and male was performed, detailing the morphological differences existing for each sex.

The development of *H. hebetor* is similar in many ways to other braconids. However, in this study, we documented the whole development of *H. hebetor*, including morphological changes, thus providing a detailed basis for the morphological characterization of the immature stages and the development of *H. hebetor*.

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