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Notes on Brood Morphology and Development of the Neotropical Eusocial Wasp *Mischocyttarus cerberus* (Hymenoptera, Vespidae, Polistinae)

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Abstract

Mischocyttarus cerberus stands out as the most investigated species of eusocial paper wasp, in Brazil. While the adult characteristics were relatively well reported in the earlier studies, very meager information was available regarding their immature stages. This study provides a general description of the immature morphology of the brood of *M. cerberus*, by studying the number of instars and analyzing the degree of influence exerted by some of the environmental factors on the individuals in the immature phases. This work involves a detailed study of 72 wasp colonies from Rio Claro and Ribeirão Preto. Using the larvae drawn from 41 nests, the number of instars was calculated; besides, the degree to which a few environmental factors could affect the immature brood development was assessed in 31 nests. Eggs showed patterns similar in terms of form and size to that of the species described earlier. The two ventral lobes, characteristic of the *Mischocyttarus* larvae, were fully developed only in the fifth instar. Based on the head measurements, we found that *M. cerberus* also express five larval instars, which is in agreement with reports published earlier for the most part of social wasps. Besides, larvae took longer than eggs and pupae to develop. From the results of our study, we concluded that *M. cerberus* showed the typical developmental pattern in the immature stages of the genus.

Introduction

The paper wasp *Mischocyttarus cerberus* is widely distributed across Brazil (Richards, 1978; Oliveira et al., 2017). One or a few inseminated females (foundresses or queens) initiate new nests, which give rise to generations of other females (workers and future queens) and males, as the cycle of the colony progresses. New colonies are established throughout the year (Giannotti, 1998a). Similar to other independent-founding wasps, no clear morphological caste differences are observed (see Jeanne, 1980). However, the nestmates establish a reproductive-based division of labor depending upon dominance-subordination interactions. Normally, only one queen exists per colony (Poltronieri & Rodrigues, 1976; Giannotti, 1998a). It lays most of the eggs and is the most aggressive female, often displays aggressive behaviors toward their nestmates (Noda et al., 2001). The adult wasps are also known to be capable of defending their nests against ants (Togni & Giannotti, 2007; Togni & Giannotti, 2008).

One common aspect of the biological and ecological studies described prior was that they focused only on the adult wasps, which seems to be the general pattern adopted in the research of eusocial insects (Giannotti, 1998; Togni & Giannotti, 2007; Togni & Giannotti, 2008). However, paper wasp colonies include substantial numbers of immature brood, like eggs, larvae and pupae. These immature stages are observed in the nest in the most part of the colony cycle and the adults often exhibit interactions with them. A considerable duration of individuals` lives is spent in

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the immature stages (Giannotti & Fieri, 1991; Giannotti & Machado, 1994; Giannotti 1995; Prezoto & Gobbi, 2005; Solis et al., 2012; Cecílio et al., 2015; Rocha & Giannotti, 2016). The considerable presence of interspecific variation in the larval morphology implies its taxonomical significance (Nelson, 1982; Kojima, 1998; Carpenter et al., 2000). Larvae of *Mischocyttarus* wasps were first described by having the first abdominal sternite with one, two or three lobes projected forward, besides their first abdominal spiracle is twice as big the remaining and mandibles have a single elongated tooth (Richards, 1978). Kojima (1998) was the first to study larvae of *M. cerberus*, however he studied only material coming from two nests and his notes regarded only to morphological aspects. Despite of that, there is still a lack of detailed information regarding the immature stages of *M. cerberus* up to this moment.

Thus, we used laboratory and field observations of the paper wasp *M. cerberus* to describe (i) the general external morphology of the eggs, larvae and pupae, (ii) the number of larval instars and (iii) the duration of the immature stage in relation to temperature and nest phase variation.

Material and Methods

Study location and wasp collection

During the years of 1991-1993 and 2017, we examined a total of 72 *M. cerberus* field colonies located in the municipalities of Rio Claro (22°24' S; 47°33' W, at elevation 612 m) and Ribeirão Preto (21°05' S, 47°50' W, at elevation 531 m), São Paulo state, Southwest Brazil. To assess the general external morphology and determine the number of larval instars, 41 field nests were collected, from which 177 eggs, 259 larvae, 31 pre-pupae (correspond to the individual in transition between fifth instar larvae and pupae) and 69 pupae were sampled. These immature individuals were preserved in 70% ethanol. To describe the duration of each immature stage, field observations were performed on a total of 31 naturally established colonies, out of which we followed the development of 404 eggs, 232 larvae and 284 pupae.

The general external morphology of immature stages

The eggs, larvae, pre-pupae and pupae were carefully studied under a binocular stereomicroscope (Leica MZ125). The immature stages were described based on the terminology used in the previous studies (Kojima, 1998; Cecílio et al., 2015; Rocha & Giannotti, 2016).

The external morphometry of the immature stages

Each immature individual was carefully removed from its cell and measured under a stereomicroscope. For each egg, we measured the maximum width, whereas for each larva, pre-pupa and pupa, we measured the maximum width of the head capsule. After measuring all samples, mean values were calculated and pair-wise comparisons were carried to check whether the groups were different or not.

The number of larval instars

The number of larval instars was determined depending upon a visual analysis of the data on head width, plotted in a frequency-distribution graph. Distinct peaks corresponding to distinct size frequency distribution peaks were categorized as instars. To validate our method, the head width data of the larvae were then tested according to Dyar's rule (Dyar, 1890), which suggested that the larval head grew in geometrical progression, increasing its maximum width in the ratio of 1.44 at each ecdysis (Parra & Haddad, 1989).

Duration of the immature stages in relation to temperature and nest phase variations

This party of the study was performed in Rio Claro. The climate classification of the area is mesothermic according to Köppen (1948), which means that there are two well defined seasons along the year, a rainy (from October to March) and a dry (from April to September) season. The eggs, larvae and pupae present in each nest were mapped during regular visits (at 2- to 10-day intervals) under natural conditions. Thus, the overall duration (in days) of each immature stage was assessed during the months of the year when the investigations were done. The months were grouped as follows: December – February; March – May; June – August, and September – November. In the discussion, we explored the manner in which the results differed with respect whether the study period was cold and dry or rainy and sunny. Besides that, duration of the immature stages was calculated based on the colonial stages of development (pre- and post-worker emergence).

Statistical analyses

The cephalic capsule width was compared using a linear model (LM). In our model, we used the cephalic capsule width as the response variable and the *M. cerberus* life stages as the explanatory variable. We used a normal model, because it best fitted our hypothesis (Weights AIC: Poisson < 0.0001; Normal $= 0.999$). Posteriorly, we used the Tukey's test for a post-hoc analysis with p correction, employing false rate discovery (*fdr*). Besides that, the developmental time for each phase (egg, larvae and pupae) was compared based on the different month groups throughout the year using the Kruskal-Wallis test. Brood development time according to nest phase (pre and post-worker emergence) were compared through Mann-Whitney U test. Statistical analyses were performed using the R and PAST version 3.20 software.

Results

The general external morphology of the immature stages

The eggs showed slight curvature, narrow at the base, fixed onto one of the nest cell wall angles and projecting into the nest cell lumen at a roughly 45°angle and coated with an adhesive secretion (Fig 1a). The vermiform larvae are soft-bodied, having sclerotized pale brown cephalic capsules, which became even darker in color through the molting cycle. The thoracic and abdominal segments are whitish, in numbers of 3 and 10, respectively (Fig 1d). Larvae are fixed by their distal end in the nest cell, until the third instar (Fig 1b); from the fourth instar onwards they become free. The fifth instar larvae has two abdominal lobes, covered by small bristles (Fig 1). The abdominal lobes are observed as small protuberances since the third instar, and they achieve full development only in the fifth instar (Fig 1d). Thus, only the last instar expresses the typical aspect of the *Mischocyttarus* larvae, with clearly evident abdominal lobes, prominently projected forward. The diameter of the first spiracular atrium (sited between the second and third thoracic segments) is almost twice the diameter of the rest of the atria (Fig 1d). The transversal anal slot is located in the last (anal) segment (Fig 1d). The head is composed of the cephalic capsule and mouth parts, which are more highly sclerotized in the last larval instar (Fig 1c). The cephalic capsule is brown in color compared to the rest of the larval body.

The pre-pupa or pharate pupa is categorized as the late period of the fifth larval instar and characterized by the elongated body of the last-instar larva (Fig 1e).Through the transparent pre-pupal cuticle the reddish-brown compound eyes of the pupa can be observed to be fully developed (Fig 1e). During the pre-pupal phase the abdominal lobes are projected rearward rather than forward. Finally, in the pupal stage, the appendages remain free from the body and the wings are not fully distended until this stage is complete (Fig 1e). At the beginning, the pupa is whitish in color with reddish-brown compound eyes, and gradually its body turns to be yellow and black. Besides, the dark pigmentation appears to develop faster than the yellow pigmentation along the body.

The external morphometry of the immature stages

The linear model analysis revealed that the groups of eggs, larvae, pre-pupae and pupae differed in their width ($F = 3365$; $p < 0.0001$). Generally, the head width increases progressively, as the wasps develop from one instar to the other (Table 1).

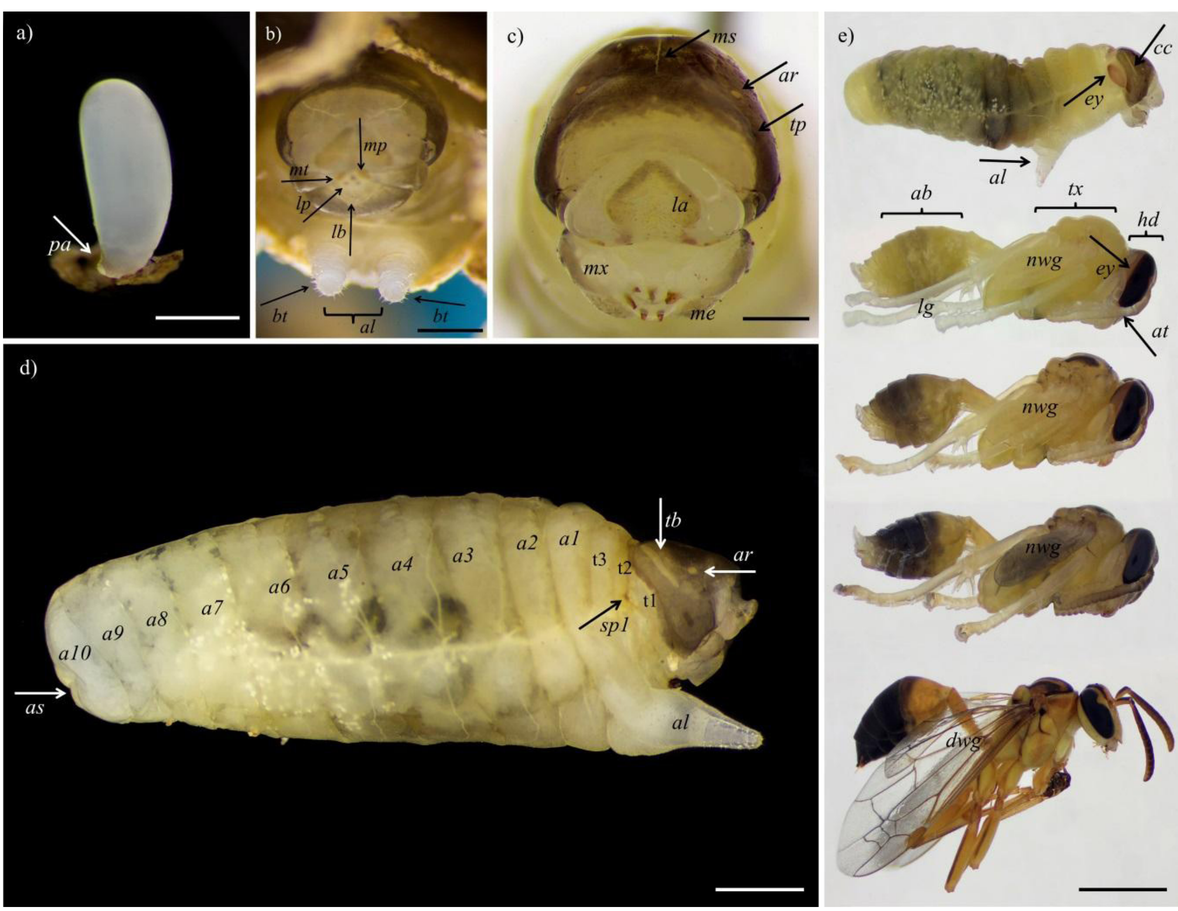

Figure 1: a) Example of one *M. cerberus* egg attached to a piece of nest material – white bar represents 0.5 mm; b) Frontal view of one fifth-instar larvae with its abdominal lobes in evidence – black bar corresponds to 1.0 mm; c) Frontal view of one fifth-instar larvae cephalic capsule – black bar represents 0.5 mm; d) Lateral view of one fifth-instar larvae – white bar represents 0.5 mm; e) Lateral view from pre-pupae with elongated body of fifth-instar larvae to a fully developed pupae before its emergence – black bar correspond to 5.0 mm. ***a1* to *a10* –** abdominal segments from 1 to 10; ***ab*** – abdomen; ***al*** – abdominal lobes; ***ar*** – antennal ring; ***as*** – abdominal slot; ***at*** – antenna; ***bt*** – bristles; ***cc*** – cephalic capsule; ***dwg*** – developed wings; ***ey*** – compound eyes; ***hd*** – head; ***la*** – labrum; ***lb*** – labium; ***lg*** – legs; ***lp*** – labial papilla;***me*** – mentum; ***mp*** – maxillary papilla; ***ms*** – median suture; ***mt*** – mandible tooth; ***mx*** – maxilla; ***nwg* –** non-developed wings; ***pa*** – point of attachment; ***sp1*** - spiracule 1; ***t1* to *t3*–** thoracic segments from 1 to 3; ***tb*** – temporal band; ***tp*** – tentorial pit; ***tx*** – thorax.

Five larval instars were determined by the visual inspection of the distribution-graph indicating the measurements of the head width (Fig 2). Supporting these findings, the five larval instars proposed expressed significant differences based on the average of their head width when compared ($F = 3365$; $p < 0.0001$). Finally, our data analyzed larval average growth rate according to Dyar`s rule , we observed mean growth ratio among the instars = 1.44 (1.39 between L1 and L2, 1.40 between L2 and L3, 1.43 between L3 and L4 and 1.52 between L4 and L5).

Table 1 – Descriptive statistics of the widths (mm) of the eggs and the cephalic capsules of the larvae (L1 – L5), pre-pupae (PP) and the pupae (P) of *Mischocyttarus cerberus.*

	N	Mean	Stand. dev	Median	Min	Max
Eggs	177	0.496	0.053	0.496	0.372	0.622
Larvae 1	49	0.452	0.041	0.465	0.346	0.558
Larvae 2	38	0.644	0.04	0.637	0.589	0.745
Larvae 3	34	0.897	0.07	0.893	0.757	1.024
Larvae 4	72	1.441	0.212	1.359	1.05	1.749
Larvae 5	66	1.895	0.099	1.884	1.756	2.148
Pre-pupae	31	1.97	0.084	1.984	1.769	2.139
Pupae	69	2.438	0.155	2.48	1.718	2.666

Duration of the immature stages in relation to monthly temperature and nest phase variations

The overall duration of each brood stage is presented in Table 2. From the Kruskal-Wallis test it is clear that the eggs ($H = 74.68$, $p < 0.001$), larvae ($H = 719.28$, $p < 0.001$) and pupae ($H = 24.37$, $p < 0.001$) take different times to develop throughout the year. The sum of the mean values of the developmental times (eggs + larvae + pupae) are similar for the warmer and rainier months (from September to May), however, they are longer for the coldest and drier ones (from June to August) (Table 2).

The mean period of incubation of the eggs in the pre-worker emergence stage of the colonies of *M. cerberus* was significantly longer than in the post-emergence stage (Mann-Whiney test, $U = 2072.5$, $p = 0.05$). The respective times of duration of the egg incubation were 13.7±4.4 (9-22, n = 17) and 11.6±3.9 (7-28, n = 191) days.

On the other hand, there was not significant difference between the larval period in the pre and the post-worker emergence stages in colonies (Mann-Whiney test, $U = 879.0$, $p = 0.68$). The respective times of duration of the larval periods were 30.2±9.7 (16-48, n = 15) and 30.5±11.9 (19-93, n = 111) days.

The mean of pupal period in the pre-worker emergence was significantly longer than in the post-worker emergence (Mann-Whiney test, $U= 1284.5$, $p = 0.001$). The respective time of duration of the pupae was 24.2±4.6 (16-33, n = 15) and 19.6±4.5 (9-36, n = 111) days.

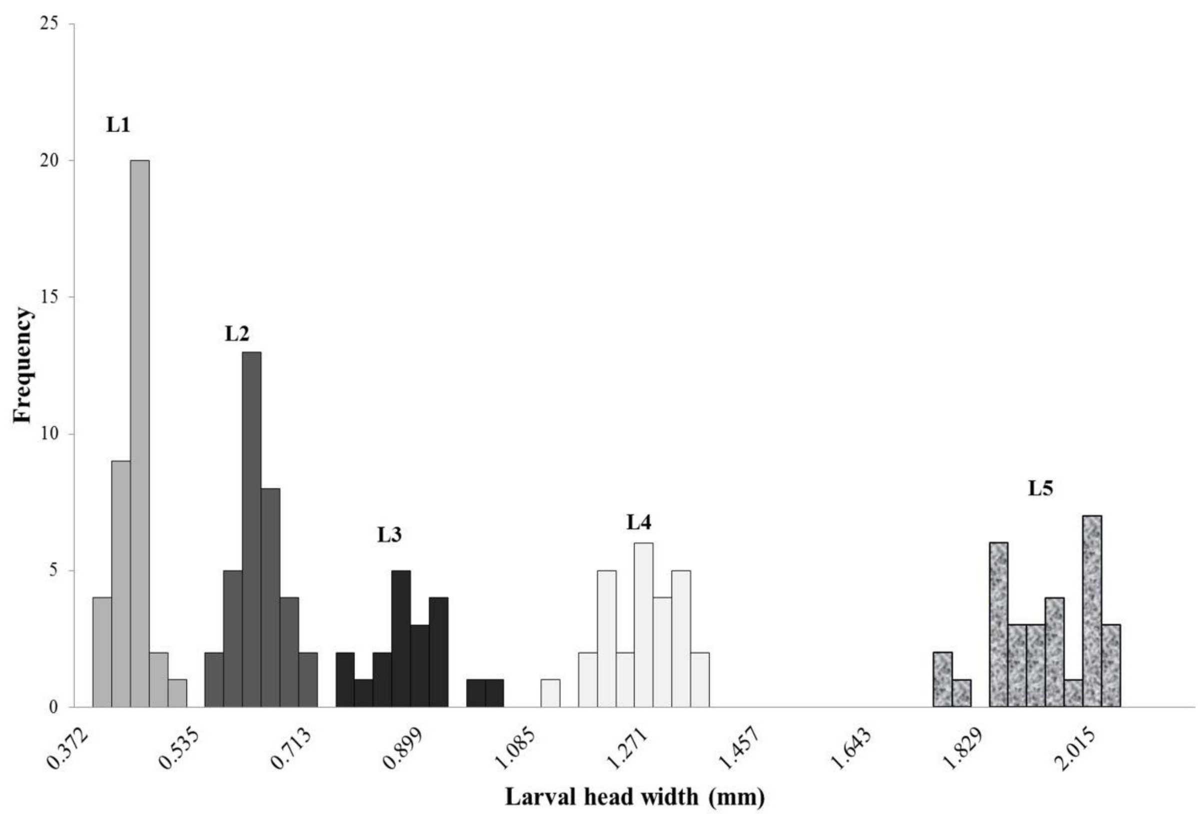

Fig 2. Frequency distribution of the maximum widths of head capsules of *M. cerberus* larvae. **L1**: first instar; **L2**: second instar; **L3**: third instar; **L4**: fourth instar and **L5**: fifth instar.

Table 2 - Average duration (in days) of the immature stages of *Mischocyttarus cerberus styx* related to the seasons.

Months	Average duration in days of immature stages			
	egg	**larvae**	**pupae**	sum on mean values
(September – November)	rainfall average 102.0 mm temperature average 20.0ºC (10.2ºC - 30.0ºC)			
	11.3 ± 3.4 (n = 100)	29.2 ± 9.0 (n = 45)	19.6 ± 3.6 (n = 29)	60.1 days
(December – February)	rainfall average 175.0 mm temperature average 20.9ºC (13.4ºC - 28.1ºC)			
	10.6 ± 2.4 (n = 233)	30.4 ± 6.2 (n = 144)	19.5 ± 2.9 (n = 169)	60.5 days
(March - May)	rainfall average 190,1 mm average temperature 19.9ºC (11.8ºC - 27.5ºC)			
	13.5 ± 4.0 (n = 60)	34.4 ± 9.4 (n = 37)	20.0±3.9 (n = 82)	67.9 days
(June – August)	rainfall average 13.3 mm temperature average 18.2ºC (7.0ºC - 29.3ºC)			
	20.9 ± 3.5 (n = 20)	69.7 ± 19.0 (n = 7)	30.3 ± 4.0 (n = 4)	120.9 days
Mean total Duration	11.7 ± 3.7	31.9 ± 10.5	19.8 ± 3.5	

Discussion

Morphology of the Immature stages

The *M. cerberus* eggs are similar in appearance to those described earlier for the wasps from the same genus (Giannotti & Silva, 1993; Cecilio et al., 2015; Rocha & Giannotti, 2016). As observed for *M. drewseni* (Giannotti & Trevisoli, 1993), only the last larval instar expressed the features of the *Mischocyttarus* larvae (Wheeler & Wheeler, 1979), in which the abdominal lobes were fully evident and prominently projected forward. From the findings of several researchers, these structures may be involved in different functions. One hypothesis suggests that they could be used as sensorial structures, based on the amount of bristles covering them (Rocha & Giannotti, 2016). A second hypothesis proposes that they could play a role when the larvae are ingesting food, functioning as a mean to hold the solid food (Reid, 1942; Wheeler and Wheeler, 1979). A third hypothesis proposes that these lobes could assist during the process of saliva transference (larvae – adult), by pumping the larval saliva into the adults when they solicit it (Jeanne, 1972), in a behavior described as "lobe erection" (Hunt, 1988). Finally, according to Giannotti & Silva (1993), these lobes may help the larvae to sustain themselves inside the cells, preventing them from falling. The pattern of the cuticle changing from a lighter color to a blackish one during the pupal stage of development appears to be the general rule when compared to the species analyzed earlier (Giannotti & Silva, 1993; Rocha & Giannotti, 2016).

Determination of the morphometry of the Immature brood and Instars

Our findings concerning determination of the number of instars corresponded to previously published results for most of the *Mischocyttarus* species, including *M. cassununga* (Giannotti & Silva, 1993), *M. drewseni* (Giannotti & Trevisoli 1993), *M. latior* (Cecílio et al., 2014) and *M. nomurae* (Rocha & Giannotti, 2016). A similar pattern has also been identified for other social wasps from different genera, such as *Agelaia* (Giannotti, 1998b), *Brachygastra* (Machado et al., 1988), *Polistes* (Giannotti, 1995; Prezoto & Gobbi, 2005), *Polybia* (Solis et al., 2012) and *Protopolybia* (Silveira, 1994). In fact, the presence of five instars has been proposed as a pattern number for the larvae of *Polistinae* and *Vespinae*, whereas for the *Stenogastrinae* the number may range from three to five (Kojima, 1998). On the other hand, our results differed from the findings of Silva (1984) and Raposo-Filho (1981), who determined only four instars for *M. extinctus* and *M. atramentarius.* Some authors proposed that the number of larval instars might vary in accordance to the biotic and abiotic factors, such as the environmental temperature, hereditary traits or even nutritional frequency (Parra & Haddad, 1989).

Like in *M. cerberus,* it was verified that in *Polistes lanio* (Giannotti, 1995) the eggs are wider than the larvae of the first instar. On the other hand, these findings differed from the results reported for the other *Mischocyttarus*, such as *M. cassunuga* (Giannotti & Fieri, 1991), *M. drewseni* (Giannotti & Trevisoli, 1993) and *M. latior* (Cecílio et al., 2015), which have eggs and first larvae instar of similar sizes. Besides that, the head width dimensions of fifth larval instars and pre-pupae were statistically different, which is supported by the findings of Rocha and Giannotti (2016). A larger pre-pupal head size might be required for providing sufficient space for the head of the pupa growing below it.

Duration of the immature stages in relation to monthly temperature and nest phase variations

On comparison with the data published earlier on the *Mischocyttarus* spp., we noticed that the larval stage is the one that takes more time to develop (Jeanne, 1972; Litte, 1977,1979, 1981; Dantas-de-Araujo, 1980; Raposo-Filho, 1981; Silva, 1984; Giannotti & Fieri, 1991; Michelutti et al., 2014; Cecilio et al., 2015). This is perhaps because larval development is affected by many more variables than only temperature, such as food availability, colony cycle and genetic factors (Silva, 1984; Giannotti & Trevisoli, 1993; Giannotti & Machado, 1994; Cecilio et al., 2015). On the other hand, the egg and pupal stages tend to be affected more by temperature throughout their developmental cycle. In other words, these two stages do not exhibit any dependence on the food provided by the foragers (Cecilio et al., 2015). The average values of brood development did not differ in most of the studied months (except from June to August), which means that studies involving brood should be avoided during the dry and cold months, because they seem to be more sensible during this period. Eggs and pupae seem to require more time to develop in pre-worker emergence nests rather than in post-worker nests, which contradicts with was previously found in other species of the same genus, where eggs develop faster in pre-worker emergence (*M. latior*: Cecílio et al., 2015). This fact might be important for colony survival, taking into consideration that as fast as the brood develop, more adult females would be in the nest to help nest-foundress and less time the foundress would be alone (especially in the cases where only one foundress start the nest) (Cecílio et al., 2015). On the other hand, it is possible to believe that it is not a problem for *M. cerberus* because cases of nest foundress association are also common (Noda et al., 2001).

In conclusion, the present study confirmed the presence of five larval instars in *M. cerberus*, which reinforces the concept of five instars as being the standard for the *Polistinae* larvae. We also added more details to the early description given for the mature larvae, by Kojima (1998). It is our hope that our data will prove useful for further studies which may involve different approaches. Considering that different larval stages were morphologically identified here, chemical analyses of the cuticle appears to be a promising field, not fully explored yet, which can be used to confirm whether the different instars exhibit specific hydrocarbon patterns, so far the only study performed up to this moment was carried by Michelutti et al. (2017). This could add another function for these chemical substances, widely used in adult-adult communication. Finally, we believe that further studies relating to the differences in types and pattern of the bristles found on the abdominal lobes are required, to clarify whether they may be used as possible taxonomic characters for the *Mischocyttarus* wasps.

Author's Contribution

RCS, ARS e EG planed, designed and executed experimental work, RCS, DSA and EG conducted data analyses, RCS, ARS, FSN wrote the manuscript.

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