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RESEARCH ARTICLE

Protocol for the in vitro rearing of *Frieseomelitta varia* workers (Hymenoptera: Apidae: Meliponini)

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ABSTRACT. In vitro rearing protocols already established for honeybees are currently being adapted to assess the risk of pesticides, and to conduct comparative developmental biology studies on stingless bees. However, differences in critical life-history traits (development time and the type of larval nutrition leading to caste differentiation process) among social bees require the development of an in vitro rearing protocol for each species and caste. We generated a protocol to produce workers of *Frieseomelitta varia* (Lepeletier, 1836), a non-endangered and highly eusocial pollinator species with wide geographical distribution. We tested the viability of using either the eggs or the first instar larvae as the starting point for in-vitro transfer. In vitro rearing was performed in acrylic plates at 30 °C and 99% relative humidity during the larval feeding phase. The humidity was subsequently reduced to 75% during the following days of development. The experimental larvae were offered either 25 µL or 27 µL of larval food. The development time, emergence and mortality rates, and morphological parameters of the emerged workers were assessed. In the process of validating the protocol, the adults that emerged after in vitro rearing were compared with colony-reared adults. In our results, 27 µL of larval food allowed 90% of workers to emerge. No significant differences were found between the emerging workers reared in vitro and those reared in the colony. The described protocol is a useful method for rearing *F. varia* workers in vitro, which can be used for diverse types of experimental approaches.

KEY WORDS. Bee development, in vitro rearing, larval food, marmelada, native bee, standardization.

INTRODUCTION

Pollination is a crucial environmental service for the maintenance of natural and agricultural ecosystems (Costanza et al. 1997, Ricketts et al. 2008, Potts et al. 2016). Among the animals that perform the pollination services, bees are essential in maintaining plant diversity, food production, seeds and pastures (Garibaldi et al. 2011, Giannini et al. 2015, Kremen 2018).

It is estimated that there are approximately 20,000 species of bees distributed in different regions of the world (Michener 2013). Meliponini stingless bees are the largest group of perennial eusocial bees. They comprise a diverse group with a pantropical distribution (Roubik 1992, Michener 2007). These bees have numerous special biological characteristics. For example, the survival of their colonies depend on a constant supply of food; their colonies are composed of a queen and dozens of workers (Grüter 2020, Michener 1974); they depend on chemical signals to recognize the individuals of the same colony and to coordinate tasks (van Zweden and d'Ettorre 2010, Leonhardt 2017). Since they are eusocial insects, they share these biological characteristics with the honeybee, *Apis mellifera* (Linnaeus, 1758), the most studied bee species.

Although stingless bees share many biological similarities with honeybees, they are unique when it comes to provisioning and oviposition (POP). In these insects, after the brood cell is filled with larval food regurgitated by the workers, the queen lays her egg on the surface of the larval food and, later, the brood cell is sealed by a worker (for more details, see review by Zucchi

et al. 1999). After the egg hatches, the larva consumes all the larval food present in the brood cell, develops and an adult bee emerges from each brood cell.

In recent years, the use of larval food from stingless bees' nests have allowed researchers to rear these bees in vitro. Laboratory rearing has generated additional knowledge that used to be impossible to obtain from colonies in nature. including the beneficial symbiotic interactions these bees have with fungi (Paludo et al. 2018), their negative interactions with bacteria (Menegatti et al. 2018) and mites (da Silva et al. 2022), and the effects of pesticides on them (Dorneles et al. 2021). Furthermore, in vitro rearing has also enabled the multiplication of colonies (Menezes et al. 2013). The possibility of rearing queens and workers in vitro has facilitated ecotoxicological, evolutionary and biological research. Additionally, it has become possible to evaluate the pollination efficiency of workers reared in colony multiplication studies and in genetic improvement programs (queen selection). A relevant contribution to this last area was the publication of an in vitro rearing method for *Frieseomelitta varia* (Lepeletier, 1836) queens from larvae at their late phases of development (Baptistella et al. 2012). However, it is necessary to develop genus and caste-specific protocols for in vitro rearing, mainly in view of the singularities in the life-history traits found in the different groups of stingless bees.

Frieseomelitta varia has a wide geographic distribution in the neotropics, including Bolivia and Brazil (Camargo and Pedro 2013), a highly social organization (Boleli et al. 1999) and is an important pollinator of wild plants and economically valuable crops in Brazil (Nascimento et al. 2012, Aleixo et al. 2013, Giannini et al. 2015). The species is classified as least concern on the endangered species list (ICMBio 2018). Envisaging evolutionary caste development studies and the use of laboratory-reared bees in diverse areas of meliponiculture, we present a protocol for the in vitro rearing of *F. varia* workers. In our study, we focused on the ideal amount of larval food necessary for the production of workers (the amount that allows for the largest number of workers to emerge), the feasibility of starting the rearing protocol with transferred eggs or larvae, development time, and the influence of in vitro production on the following morphological parameters: body weight and head width of emerged bees compared to those of bees emerged naturally in the colony.

MATERIAL AND METHODS

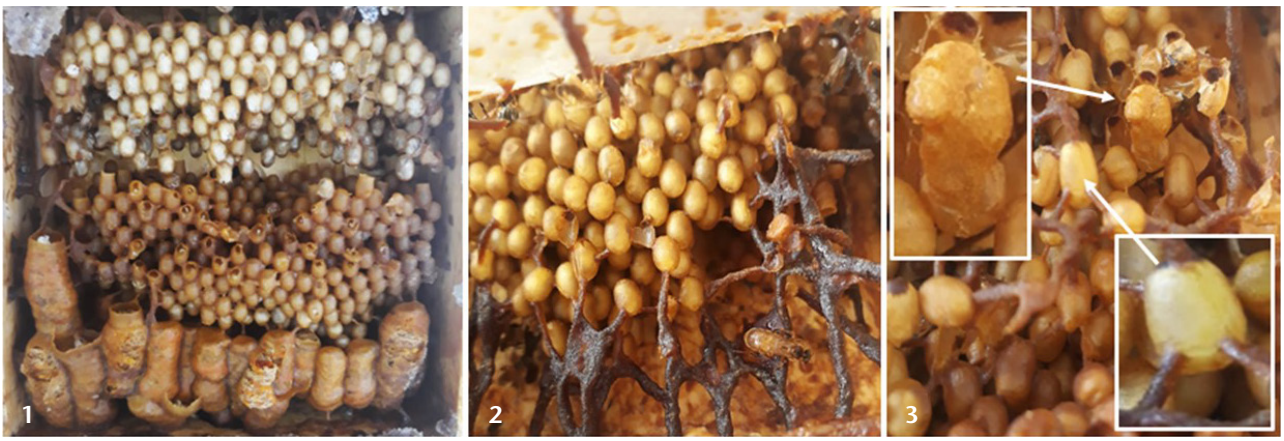
Study system

In *F. varia*, our study model, the brood cell sets take the form of clusters. The queens, solely responsible for laying eggs in the colony, develop from the union of two cells of equal size, that is, a larva consumes larval food from its own brood cell and from a neighboring cell. On the other hand, if this process does not occur, the larva will give rise to a worker (Figs 1–3). *Frieseomelitta varia* bees are midsized (0.5 mm) and live in perennial colonies with approximately 1,200 individuals.

The experiments were conducted at the Federal University of Alfenas (Minas Gerais, Brazil), in the urban center of the city (21°25'14.6"S, 45°56'52.8"W). The eggs and larvae were obtained from colonies of *F. varia* established in our Experimental Meliponary in 2018.

Collection and storage of larval food

For the in vitro rearing, food from recently constructed brood cells in the colonies was used. The cell was identified as being recently constructed by the dark brown color of its walls. Wood modules with recently constructed brood cells were removed from and taken to the laboratory. The cells with eggs were opened with tweezers and the eggs were removed. Then, the food was collected with an automatic micropipette (100 µL)



Figures 1–3. Photo of a representative healthy *Frieseomelitta varia* nest used to collect larval food for in vitro rearing experiments showing: (1) the interior of the *F. varia* nest with brood cells and pollen pots; (2) the shape of the brood cells in clusters; (3) arrow A indicates a brood cell of a queen developing in the nest and arroe B indicates a brood cell of a worker. Note that the size of both cells are different sizes.

and transferred to a 1 mL microtube. After collecting the food, the microtube was stored at -20 °C until the food was used. Food collection from the three colonies, established as replicates, was carried out every 15 days. During collections, not all brood cells from colonies containing eggs were removed, leaving them intact so that new individuals could develop and emerge. This avoided weakening the colonies during the experiments.

Volume of larval food, transferring and rearing conditions

For the experiments, 96-well ELISA-type U bottom acrylic plates were used. Each cell was 0.7 x 10 mm (400 µL volume). During the transfer of eggs or larvae it is not possible to distinguish the brood from which workers, queens or males will emerge. However, after the bees emerged, we verified the developed corbiculae, which signals that they are workers. To evaluate the viability of transferring eggs for the in vitro rearing of workers, 25 or 27 µL of larval food were placed into the cells of acrylic plates using a micropipette (100 µL). Twenty seven microliters was chosen as the greater amount based on the average volume of larval food found in natural cells that result in workers (Baptistella et al. 2012). Then, newly constructed dark brown brood cells were uncapped and the eggs were transferred with the help of a transfer needle. They were carefully deposited onto the larval food. For each tested larval volume (25 and 27 µL), three acrylic plates were used, with three repetitions in each one (triplicate), containing 10 eggs transferred from three colonies, totaling 90 eggs. In addition to egg transfer for viability assessment, first instar larvae (newly hatched, L1) were also transferred to acrylic plates using the same method used for egg transfer. In this experiment, for each larval food volume, three acrylic plates were used, with three replications and each containing 10 larvae, totaling 180 larvae for each larval food volume. The plates with eggs or larvae were put into plastic boxes (30 cm long and 22 cm wide) sealed with PVC film and capped. For each experiment, plates containing eggs or larvae were placed individually in plastic receptacles. The number of eggs and larvae used in our tests allowed assessment of the feasibility of the protocol and kept the colonies with enough brood. Thus, the eggs and larvae transfer experiments were not carried out simultaneously and the same colonies were used for both food collection and as a source of eggs and larvae.

The humidity during the rearing process was controlled according to Menezes et al. (2013). All samples were placed in a BOD incubator (biochemical oxygen demand) at 30 °C and 99% relative humidity during the larval feeding phase. The humidity was reduced to 75% during the following days of development. A saturated solution of sodium chloride in a plastic pot inside the incubator was used to control humidity. The solution was replaced every seven days to avoid any kind of proliferation of microorganisms, so that the health of larvae and eggs in the experiments was not affected. Physical parameters were recorded with a thermohygrometer. Daily, while the transferred workers were monitored under a stereomicroscope (Carl-Zeiss-Promenade 10), the dark colored dead individuals were removed from the plates with the aid of cotton swabs.

During the in vitro experiments, we observed that most larvae transferred from both groups (25 and 27 µL of larval food) did not completely consume the food offered to them during a period of 5.3 ± 0.75 days (mean ± SD), leaving some remnants in artificial cells. After the feeding phase, the larvae started the defecation phase 9 ± 1 days (mean ± SD), and after that, a proliferation of fungi was observed, causing the mortality of some workers. To reduce the mortality rates due to microorganism proliferation on larval food debris and larval feces, the developing workers were transferred to new clean and sterilized acrylic plates with the aid of a toothpick with a string loop at one end (Figs 4–8). This adapted toothpick, unlike the tweezers, did not injure the larval bodies at the time of transfer and, consequently, there was no mortality of larvae after this method was defined (Figs 9–12).

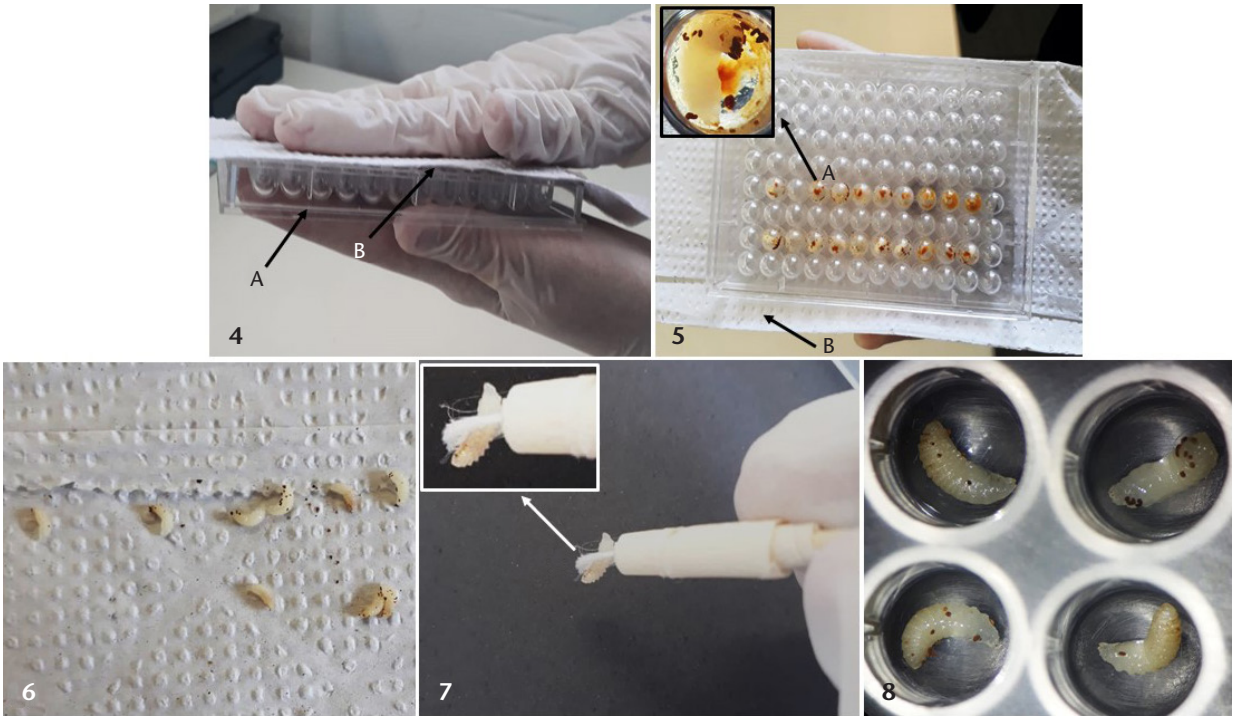
Parameters for protocol validation

After standardizing the larval food volume and the viable developmental stage for the production of workers, based on the birthrate of larvae and emergence of workers, some parameters were used to validate the in vitro protocol, such as defecation time, development time, number and percentage of emerged adults. These variables were observed and recorded daily.

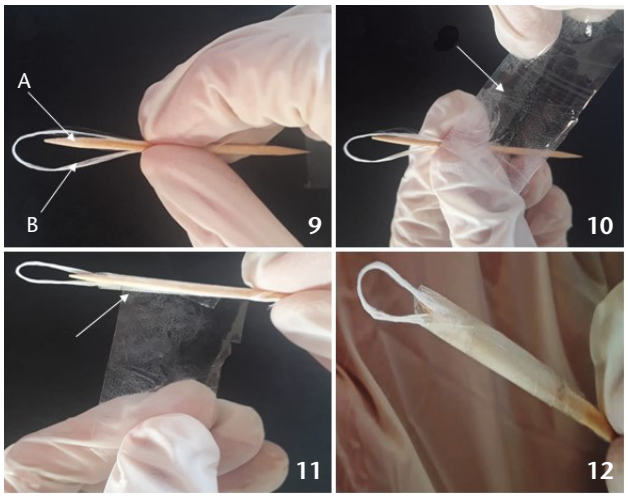
The weight and head width of individual bees that emerged in vitro, and of those naturally born in the colony, were analyzed to compare their similarity and to verify if there were morphological changes caused by the in vitro rearing. To make the comparison, 10 brood cells that were light yellow, with bees at the Pbd developmental stage (brown-eyed pharate-adult, dark pigmented cuticle; Hartfelder et al. 2006), were carefully removed from three colonies (totaling 30 bees), taken to the laboratory, and put under the same temperature and humidity conditions of the experiments until the adults emerged. Thirty workers reared in vitro were also measured. The weight of the in vitro and naturally emerged workers was accessed 24 hours after emergence with the help of an analytical balance (Brand ACCULAB model ALC – 210.4). The same bees were used to analyze the maximum head width. The bodies of the workers were adjusted and put in Petri dishes on a black background and photographed with a digital camera coupled to a microscope (Carl-Zeiss). Using the photographs, the maximum head width was measured with the software ImageJ version 1.8. Further, the in vitro development time of a total of 30 workers (10 bees/experiment) was also monitored daily, but only for the group of larvae receiving the larval food volume that presented the highest number of emergent workers. This is the reason why this parameter was observed only for workers produced in vitro; it is difficult to monitor the development of bees inside their brood cells.

Statistical analysis

The data were analyzed in an R environment (R Core Team 2016). The assumption of normality was assessed using



Figures 4–8. Procedure to transfer larvae to new clean and sterilized acrylic plates: (4) preparation of larvae for transfer, arrow A indicates an acrylic plate on which *F. varia* workers were produced and arrow B indicates paper towels; (5) acrylic plate with wells facing down and paper towel, arrow A indicates larvae inside the plate wells with the presence of larval food remains during the defecation period and arrow B indicates paper towels; (6) Larvae transferred to paper towel; (7) arrow indicates collecting larvae with an adapted toothpick. The size of the toothpick is 7 mm and the tape is 10 mm; (8) larvae transferred to clean and sterilized acrylic plates.



Figures 9–12. Creation of a larval capture device: (9) arrow A indicates the toothpick and B indicates the antiseptic string or line fitted to the toothpick; (10) arrow indicates an adhesive tape (any tape can be used); (11) arrow indicates the tape was wrapped around the toothpick, securing the thread; (12) larvae capture device (larvae are C-shaped and fit perfectly with the line capture method).

the Shapiro-Wilk test. Normal data were subjected to analysis of variance (ANOVA), and those that did not show normal distribution were subjected to the Kruskal-Wallis H test. Probability values were considered significant when less than 5% ($p < 0.05$).

RESULTS

The viability of transferring eggs for in vitro rearing

When eggs were transferred to 25 µL of larval food, an emergence rate of 12% was obtained, and an immediate mortality rate of 88% of the eggs was recorded after the transfer (n = 90 eggs). When the volume of larval food was 27 µL, the mortality rate climbed to 90% of the eggs (n = 90 eggs). The data regarding egg survival after transfer, and worker emergence in the two larval food volume experiments were not normally distributed, and indicate that there are no statistical differences between them. In other words, whether the volume of larval food was 25 or 27 µL did not make a difference in either egg survival or number of emerging workers ($\chi^2 = 4.857$, df = 4, p = 0.302).

The viability of transferring early larvae for in vitro rearing

We tested the viability of the first instar larva for the in vitro production of *F. varia* using volumes of 25 and 27 µL of larval food

(Table 1). When the larvae were transferred to 25 µL of larval food, a total of 53 workers emerged (n = 180 larvae). In the experiment using 27 µL of larval food, 89 and 97% emerged as adults (n = 180 larvae). The Kruskal-Wallis H test indicated that there were no statistical differences between the two larval food volumes when it comes to worker emergence (χ^2 = 11, df = 8, p = 0.201).

The Shapiro-Wilk test indicated that the survival and mortality of the larvae followed a normal distribution, respectively (W = 0.862, p = 0.053). Analysis of variance (ANOVA) indicated a significant difference in survival and mortality between the group of larvae fed 25 and those fed 27 µL of food, respectively (F = 13.45, p <0.004) (Table 1). The survival rates of the larvae fed 27 µL of food was higher, with only 10 and 14% mortality (tests 1 and 2, respectively), while the survival rates of the larvae fed 25 µL of food was lower (66 and 56%).

Table 1. Transfer of *Frieseomelitta varia* larvae to produce workers in vitro using different quantities of larval food (µL) and their respective survival rates (%).

Test	Volume of larval food (µL)	Transferred larvae	Larval survival rate		Emergence rate of workers		Mortality rate of larvae	
			Number	(%)	Number	(%)	Number	(%)
1	25	90	60	66	22	36	30	33
2	25	90	51	56	31	60	39	43
Total		180	111		53		69	
3	27	90	81	90	72	89	9	10
4	27	90	77	85	75	97	13	14
Total		180	158		147		22	

Morphology of in vitro and naturally emerged workers

The head width and body weight of workers that emerged from larvae fed 27 µL of larval food (the experiment that resulted in the highest rate of emerged bees, n = 81% workers), were compared with the head width and body weight of colony-born workers. There were no significant differences in the weight of workers produced in vitro and bees produced naturally (χ^2 = 49.49, df = 42, p = 0.198). The Shapiro-Wilk test indicated that the body weight of bees reared in vitro and naturally did not follow a normal distribution (W = 0.928, p = 0.001). The mean weight obtained from the in vitro experiment was 0.098 SD 0.015 mg, and from the colony-born bees it was 0.0146 SD 0.028 mg. The individual analyzes of the head width of workers produced in vitro (n = 30 workers) and in the colony (n = 30 workers) indicated that there are no significant differences (χ^2 = 10.64, df = 10, p = 0.385). The Shapiro-Wilk test also indicated that the maximum head width of workers reared in vitro and in colonies did not follow a normal distribution (W = 0.944, p = 0.008). The in vitro workers had a mean head width of 0.22 SD 0.2 mm and individuals emerged in colonies had a mean head width of 0.23 SD 0.1 mm.

Development in vitro

We verified that the development cycle of *F. varia* workers from the first larval instar until adult age (Figs 13–19) has a mean of 33.3 SD 0.57 days in duration. The Table 2 shows the timing of events that occurred during the post-embryonic developmental stages of *F. varia* workers produced the experiment using 27 µL of larval food. Figures 13–19 shows the characteristics of the individual development at each stage according to the color of the body.

DISCUSSION

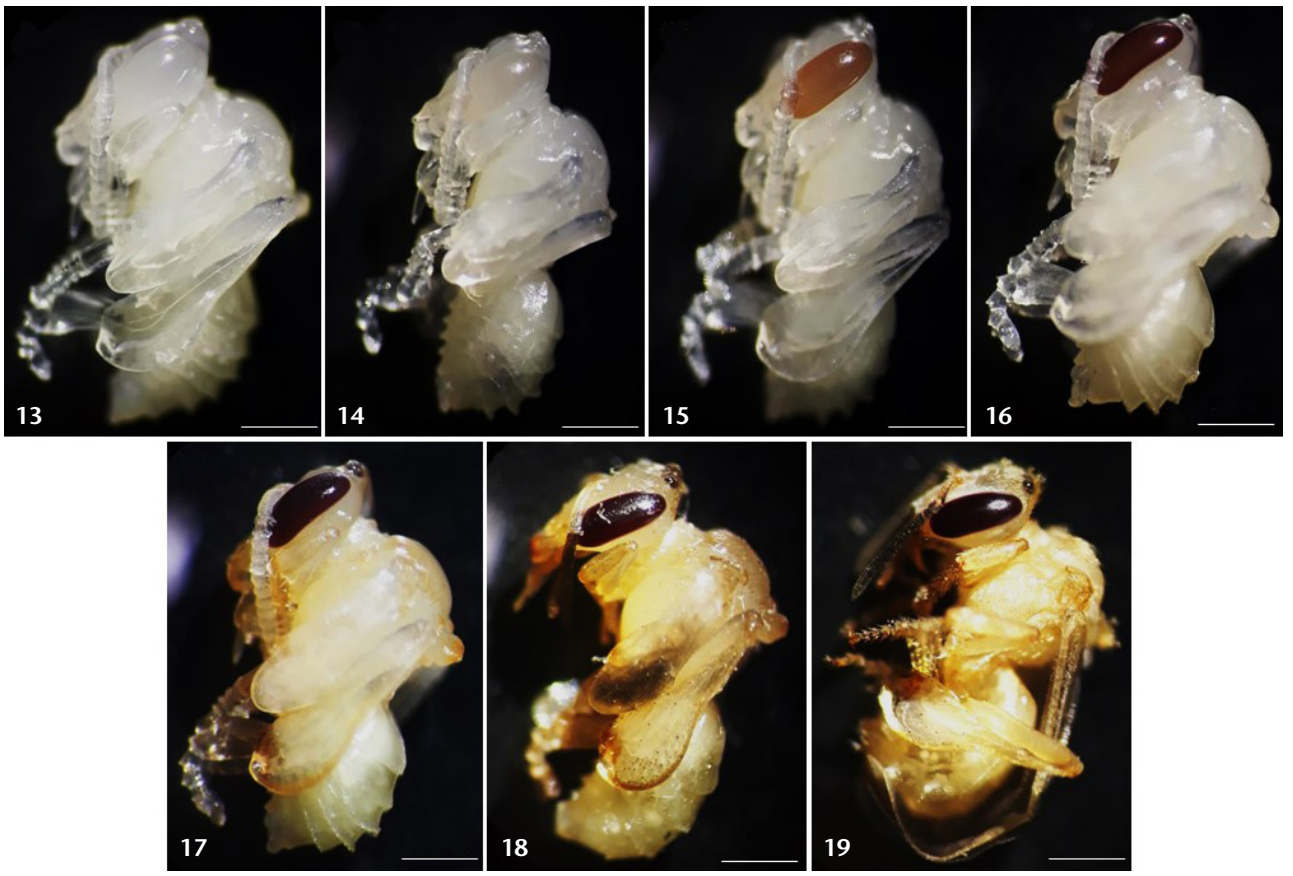
In our results, transferring eggs to in vitro resulted in a low rate of emerging adult workers, regardless of the amount of food provided. According to Velthuis and Velthuis (1998), inside the brood cells, larvae and eggs need to maintain contact with the air to breathe. The eggs in a vertical position come into contact with the food for only one seventh of their length and approximately 5% of their volume. In our study, when the eggs were transferred to the larval food on the acrylic plates, they probably submerged in the larval food. Another explanation for the high mortality rate of transferred eggs may have to do with developmental singularities of bee's eggs. Unlike the eggs of other insect orders (e.g., *Drosophila* spp.), the eggs of bees seem to be highly sensitive (DuPraw 1961, Buttstedt et al. 2018). They only develop in the upright orientation (this notion even prompted the development of protocols for egg rearing; Wegener et al. 2009). In any case, the high mortality rate registered during our experiments (25 µL = 88%; 27 µL = 90%) suggests that it is unfeasible to start from eggs to rear workers in vitro.

The experiments transferring the first instar larvae resulted in high rates of larval survival and worker emergence. In the four experiments carried out using 25 and 27 µL of larval food, we recorded significant differences in larval survival and mortality rates. The two repetitions on 27 µL of food resulted in low mortality, with an emergence rate of 89 and 97% (n = 81% workers). However, when 25 µL of larval food were provided,

Table 2. Timing of developmental events during the in vitro rearing of *Frieseomelitta varia* workers. Pupal staging was based on the intensity of eye and cuticle pigmentation, as suggested by Hartfelder et al. (2006).

Experiment	Feeding duration (days)	Defecation	Pre-pupa	Pupa eye white	Pupa eye pink	Pupa eye light brown	Pupa eye dark brown	Time until emergence (days)
1	5	9	12	16	18	19	24	33
2	6	8	12	16	18	21	25	34
3	5	10	12	18	20	23	25	33
Mean	5.3 SD 0.75	9 SD 1	12 SD 0	16.6 SD 1.15	18.6 SD 1.15	21 SD 2	24.6 SD 0.57	33.3 SD 0.57

*The ordinal numbers indicate the exact days that the events occurred (10 bees/experiment).
SD indicates the standard deviation of the mean value.



Figures 13–19. Development phases of *Frieseomelitta varia* reared in vitro: (13) Pw: white-eyed pupa, unpigmented cuticle; (14) Pp: pink-eyed/pharate-adult transition, unpigmented cuticle; (15) Pb: brown-eyed pharate-adult, unpigmented cuticle; (16) Pbl: brown-eyed pharate-adult, light pigmented cuticle; (17) Pbm: brown-eyed pharate-adult, intermediary pigmented cuticle; (18) Pbd: brown-eyed pharate-adult, dark pigmented cuticle; (19) recently emerged (all pupae incubated at 30 C and 75% humidity). Developmental phases modified from Hartfelder et al. (2006). Images made with a Carl-Zeiss microscope. Scale bars: 13–18 = 4 mm, 19 = 5 mm.

only 47% of the individuals became workers, with high larval mortality. These results are consistent with the hypothesis that there is a precise regulatory system controlling the amount of nutritional input triggering metamorphosis (Nijhout et al. 2014).

The establishment of a protocol for in vitro rearing is the first step to obtain a standardized method and its subsequent use in other types of studies. Our results suggest that the provision of 27 µL of larval food and the use of first instar larvae can be adopted by different types of studies, including exploring the effects of pesticides and diseases on the life-history of in vitro workers, nutrition and development, production of new colonies, and evaluation of the pollination efficiency of associated cultures (e.g., bell pepper Giannini et al. 2015, sweet pepper Nascimento et al. 2012), among others.

After succeeding in obtaining high rates of survival using our protocol for in vitro rearing, we measured two critical biological parameters in the emerged adults and compared them with the same parameters in colony-born bees. Workers offered 27 µL of larval food did not show significant differences in body weight and head width compared to workers that emerged naturally. This means that the environmental parameters and handling did not cause morphological changes. Moreover, the duration of each developmental stage of bees fed 27 µL of larval food were not very different from L1 to adulthood (33.3 ± 0.57 days). The brood cells of *F. varia* have a cluster-shaped structure (brood combs), and during the process of uncapping the cells to transfer larvae to the plates, it was observed that the eggs in the upper positions of the nest hatch first than the eggs in the center of the comb. Thus, the slight differences registered in developmental time might simply reflect the timing of queen oviposition and subsequent larval hatching. These differences in the development time of workers do not invalidate the proposed rearing method. For comparison, Dorigo et al. (2019) also observed changes in the development time of *Melipona scutellaris* (Latreille, 1811) workers. In our study, several workers emerged. We recommend that, in the future, studies are conducted to

explore how the volume of larval food influences the behavior and acceptance of the individual produced in vitro by other bees in the colony (queens and older workers). These parameters were tested in other studies, for example, for queens (Baptistella et al. 2012, Fernando dos Santos et al. 2016).

Although studies trying to establish standardized methods to rear stingless bees for different purposes are being developed and adapted with satisfactory results, the standardization of the in vitro rearing protocol for the stingless bee *F. varia* has several advantages. First, according to the Red Book of Brazilian Fauna Threatened with Extinction, released in 2018 by ICMBio (Chico Mendes Institute for Biodiversity Conservation), *F. varia* was categorized as Least Concern (LC), while other species used for in vitro standardization such as *M. scutellaris* are at risk of extinction in the wild (EN). Another important point to consider is that *F. varia* is somewhat not aggressive and colonies can be easily handled and purchased from stingless beekeepers.

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JAS collected larval food and biological samples; JAS conducted the experiments and analyzed the parameters for protocol validation; JAS, ARB and MW analyzed the data and were responsible for writing the article.

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The authors have declared that no competing interests exist.

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