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The behaviour of laying workers and the morphology and viability of their eggs in *Melipona bicolor bicolor*

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Abstract. As in many other stingless bees, *Melipona bicolor bicolor* Lepeletier (Apidae: Meliponinae) workers lay two morphologically distinct types of eggs: slender ones that have a typical patterned chorion, and larger ones that lack this pattern. In this paper we report on the relation between egg morphology and the behaviour of the workers that lay such eggs. In most cases, the laying of each of these egg types is accompanied by a unique sequence of behaviours. After a worker has laid the unpatterned type of egg, she generally leaves the cell, giving the queen the possibility of eating this egg. In the case of the patterned egg type, the worker usually closes the cell immediately after her egg laying. When worker egg laying occurs right after a series of regurgitations, it stops the queen from ovipositing. When, instead, a worker lays an egg after queen oviposition, the cell contains two eggs. This study also revealed cases in which workers laid slender, patterned eggs without closing the cell, and other cases where workers laid large, spherical, unpatterned eggs and instantly closed the cell. Experiments in which worker eggs, destined to be eaten by the queen, were protected by covering the cell artificially with a piece of wax showed that some of these eggs developed into larvae, although they were occasionally relatively small. The occurrence of a range of combinations of egg-laying behaviours and egg morphologies in *M. b. bicolor* workers is discussed from the perspective of worker egg laying in other stingless bees.

Key words. Egg morphology, egg viability, laying worker behaviour, *Melipona bicolor bicolor*, reproductive eggs, stingless bees, trophic eggs.

Introduction

Colonies of most species of stingless bees (Apidae) are headed by a single, singly mated queen that lays most of the eggs (Peters *et al*., 1999). The queen lays her eggs during the so-called 'provisioning and oviposition process' (Sakagami *et al*., 1965), and the haplo-diploid system of sex determination, characteristic of Hymenoptera, makes it possible for her to choose whether to produce a female or a male egg. Permitting or withholding sperm, stored in the spermatheca, to fertilize an egg during its passage along the oviduct makes this option possible (Michener, 1974).

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In provisioning and oviposition processes a cell is filled with liquid food, which is a mixture of nectar and pollen regurgitated by a number of workers, often within a period of several minutes. Thereafter, the queen lays her egg quickly on top of this liquid and a worker closes the cell (Sakagami, 1982). After hatching, the larva will eat the deposited food and pupate. In this way a single individual will emerge from each cell.

Brood care, nest defence and food collection are carried out by the workers, females which are normally uninseminated (Sakagami, 1982). However, workers of many species of stingless bees can produce male offspring. In most species, workers lay eggs after a brood cell has been filled with larval food and the cell is not yet oviposited in by the queen. In general, worker eggs are eaten by the queen, therefore they are referred to as trophic (Sakagami & Zucchi, 1963; Sakagami, 1982). In some cases a worker lays an egg that escapes being eaten by the queen, which thereafter develops into a male

(Beig, 1972; Contel & Kerr, 1976; Machado *et al*., 1984; Bego, 1990; Koedam *et al*., 1999; Sommeijer *et al*., 1999). Such eggs are referred to as reproductive.

The laying of each type of worker egg is accompanied by a typical behavioural sequence: after a worker has deposited a reproductive egg she closes the cell without delay. In the case of trophic egg laying, the worker leaves the cell after oviposition, giving the queen the opportunity of eating and replacing the worker egg with her own egg (Sakagami, 1982).

When a worker lays an egg and immediately closes the cell, she faces opposition by the queen but offers resistance, thus preventing the queen from eating the worker-laid egg, and then also excluding her from laying an egg herself (Koedam *et al*., 1999). When such worker egg laying occurs after the queen's oviposition, the worker experiences no opposition, resulting in a cell with two eggs. For *Scaptotrigona postica*, Beig (1972) found that when a cell contains two eggs, the larva that hatched from the worker-born egg will kill the queen-born egg or larva. Consequently, a worker-derived male emerges.

Apart from behavioural differences, trophic and reproductive worker egg laying in stingless bees seems to be associated with the occurrence of two distinct forms of worker eggs: slender and larger, more spherical ones. As was found in *Melipona rufiventris paraensis* (Sommeijer *et al*., 1984), the two types of worker eggs differ as to morphology. The slender eggs have a typical network patterning, whereas the larger ones lack this patterning (see also Koedam *et al*., 1996). Studies of Akahira *et al*. (1970) and Cruz-Landim & Cruz-Höfling (1971), both on *Trigona (Scaptotrigona) postica*, revealed that in this species trophic eggs probably lack the possibilities of developing into adults.

The current use of the terminology 'reproductive' and 'trophic' as regards worker egg laying in the stingless bee literature is somewhat confused. The concepts are normally used to differentiate between egg types but by definition the word 'reproductive' (egg potency) does not exclude the word 'trophic' (egg use). In our view, worker behaviour when laying eggs, the morphology of the deposited eggs and their viability should be treated separately.

Melipona b. bicolor has a special status among stingless bees in that it is facultatively polygynous: one or more physogastric queens can be found in the same colony. Moreover, in *M. b. bicolor*, virgin queens are tolerated to a considerable extent (Bego, 1989). Not only can they often be seen freely walking around in the nest, but they sometimes, similar to workers, stand very close to one of the egg-laying queens, especially during provisioning and oviposition processes. All this makes it possible that in *M. b. bicolor* a more egalitarian dominance relationship among colony members exists than in, for example, *M. favosa*.

In this paper we present data on worker egg laying in *M. b. bicolor*. For this we observed the sequences of egg-laying behaviour performed by individual workers; we examined the morphology of their eggs and we determined worker egg viability. We view the results from the perspective of worker egg laying as it occurs in other stingless bees.

Materials and methods

Over a period of one and a half months the behaviour of laying workers was studied in the facultatively polygynous stingless bee species *Melipona bicolor bicolor* Lepeletier. By collecting and microscopically examining freshly laid worker eggs, the relationship between the behaviour of the laying workers and the morphology of their eggs was studied. Three free-foraging colonies of *M. b. bicolor* that were housed inside the bee laboratory of the Institute of Bioscience, University of São Paulo, were used. When the investigations started, two physogastric queens were present in two of the three colonies and each of them was active in egg laying. In one of these colonies one of the queens disappeared after 15 days, the colony thus remaining monogynous. In another colony both queens were superseded after 26 days. From that moment onwards a young attractive queen was seen in the colony but was not yet active in egg laying. The third colony had one physogastric queen during the entire observation period.

To study the relation between egg-laying behaviour and egg morphology, deposited eggs were removed for analysis shortly after oviposition but not before it was clear what the next step of the laying worker would be, whether she was also going to close the cell or leave it, leaving the egg to be devoured.

The method applied for preparing eggs for scanning electron microscopy has been described in Koedam *et al*. (1996).

To study the relation between egg-laying behaviour and egg viability, some cells in which workers oviposited and which were subsequently sealed were opened, several days later, to inspect whether the larva had hatched. Other cells were provisionally closed with pieces of wax immediately after queen or worker egg laying. Workers transformed these pieces of involucrum into a proper cell lid. By daily inspection of the peculiarities of the morphology of these lids it was possible to eliminate those cells from our trials that were re-opened and provided with another egg in our absence. In the case of queen oviposition, this experiment served to test the extent to which workers were provoked to open cells that were artificially closed.

Results

Worker egg laying and egg morphology

There were two sequences of worker laying behaviour; after her egg laying a worker could either immediately rotate (folding the cell rim inwards) to close the cell or she could leave the cell. The former sequence could occur before the queen was able to oviposit as well as following the queen's oviposition. Leaving the cell exclusively occurred before the oviposition of the queen in the same cell. In 46 of the 68 cases of laying behaviour that occurred while the queen was waiting near the edge of the cell rim, the worker abandoned the cell after her egg laying (67.7%, Table 1). In the other cases, the worker started to close the cell after her oviposition in it. In the colony with one physogastric queen, on the four occasions that a worker oviposited in a cell while the queen was waiting near

Table 1. An overview of the different types of worker eggs, patterned, unpatterned and intermediate patterned, laid in queenright colonies and under orphan conditions. A worker could lay her egg immediately after a series of regurgitations and start to seal the cell, thereby excluding the queen from laying her egg (Before queen), or this worker could, after oviposition, flee from the cell, thereby giving the queen the opportunity to consume the egg. A worker could also lay her egg after the oviposition of the queen in the same cell, thus resulting in a cell with two eggs (After queen). Note that in all cases the deposited egg was removed for analysis shortly after oviposition but not before it was clear whether this worker was also going to close the cell or whether she had abandoned it, leaving the egg to be eaten

	Queen(s) present				
Colonies	Before queen		After queen	Orphan	
Egg laying of the worker					
Subsequent worker behaviour	Close cell	Flee	Close cell	Close cell	Flee
Morphology worker eggs					
Patterned	19	3	13	5	11
Unpatterned	3	41	–	–	5
Intermediate pattern	–	2	–	1	11

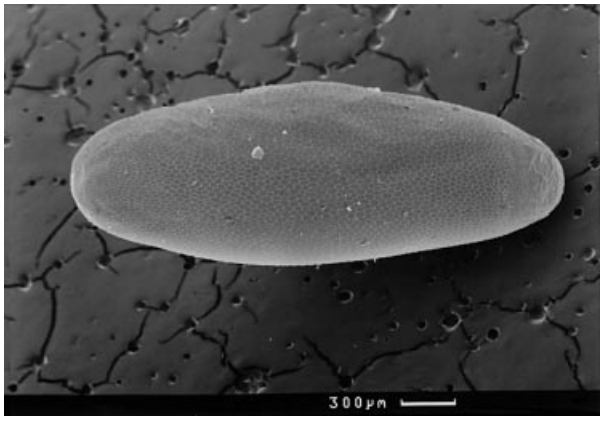

Fig. 1. SEM image of a patterned worker egg that was collected from a queenright colony of *M. b. bicolor*. Towards the posterior side there is an abrupt transition from the reticulate pattern to the smooth basal apex.

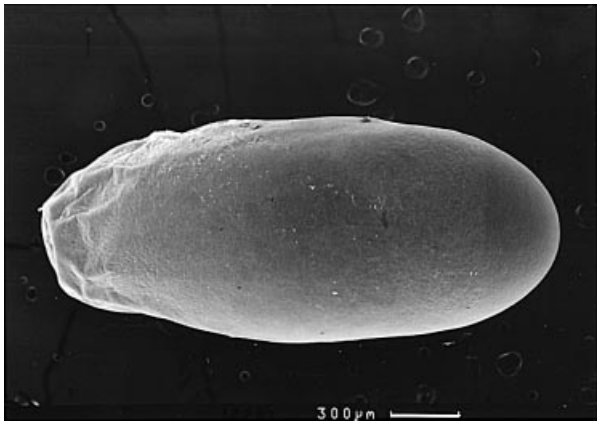

Fig. 2. SEM image of a worker egg, collected from a queenright colony of *M. b. bicolor*, which lacks the polygonal network pattern.

its edge, the worker also tried to close the cell. Only in this colony workers also oviposited after the queen had placed her egg in the cell (n = 12 cells, 13 worker eggs). Twelve of these ovipositions were accompanied by immediate cell-closure by the worker that laid the egg, resulting in a brood cell containing two eggs. In one case, we observed that two workers each placed an egg into the cell, the second worker pushing aside the first worker, which resulted in the presence of three eggs standing upon the larval food.

Of 114 worker eggs that were collected and microscopically investigated, 51 showed a reticulate chorion pattern (Fig. 1), 49 lacked this pattern (Fig. 2) and 14 showed intermediate levels of chorion patterning. Patterned eggs always had a smooth posterior pole, the part which is normally in contact with the liquid inside the cell. Under light microscopy, eggs without a reticulate chorion patterning sometimes revealed patterning at the anterior pole around the micropyle area. In addition to this, the intermediate eggs had regions of very weak patterning, which were often conspicuous near the posterior pole in the zone where, in patterned eggs, the patterning abruptly changes into the bold posterior pole (Fig. 3). Under scanning electron microscopy, this weak patterning could not be detected, which may be due to loss through the fixation procedure.

Worker eggs lacking a polygonal pattern covering the egg surface were significantly bigger than those worker eggs that were patterned (unpatterned: 3.12 ± 0.19 × 1.29 ± 0.08 mm, mean ± SD, n = 49; patterned: 2.99 ± 0.10 × 1.07 ± 0.05 mm, mean ± SD, n = 51, Fig. 4). Intermediate patterned eggs were similar in length and width to unpatterned eggs (3.12 ± 0.13 × 1.27 ± 0.07 mm, mean ± SD, n = 14; ANOVA, length: F = 10.0, P < 0.001, d.f. = 2; width: F = 147.2,

$P < 0.001$, d.f. = 2, $N_1 = 51$, $N_2 = 49$, $N_3 = 14$, Scheffé F-test ($\alpha = 0.05$): N_1 vs. N_2 and N_1 vs. N_3 significant, N_2 vs. N_3, non-significant).

Our results also reveal that in *M. b. bicolor* the two behavioural sequences of egg-laying behaviour are not always related to egg morphology as has been reported for other stingless bees. We found that in the case of the laying of patterned eggs, on 37 of 51 occasions this was followed by worker cell closure, whereas with eggs without a pattern, in only three of 49 cases was this followed by cell closure (Table 1). Of the 14 eggs laid by workers having an intermediate chorionic pattern, only once did a worker close the cell after oviposition.

Only in the colony having one physogastric queen did the relation between the behaviour of laying workers and the morphology of their eggs reveal a clear pattern. When workers left the cell after oviposition, all eggs lacked the polygonal chorion pattern on the surface ($n = 15$), whereas when workers started to close the cell immediately after oviposition, all eggs were patterned ($n = 17$). In this colony the former ovipositions happened directly after a series of worker regurgitations of larval food into the cell.

In addition, in one colony workers continued producing eggs after the replacement of the two physogastric queens. In the 18 days after the supersedure, newly oviposited cells appeared only at a low rate. During this period, 12 intermediate eggs were collected and, in 11 of these cases, the ovipositing worker left the cell after egg laying (see Table 1).

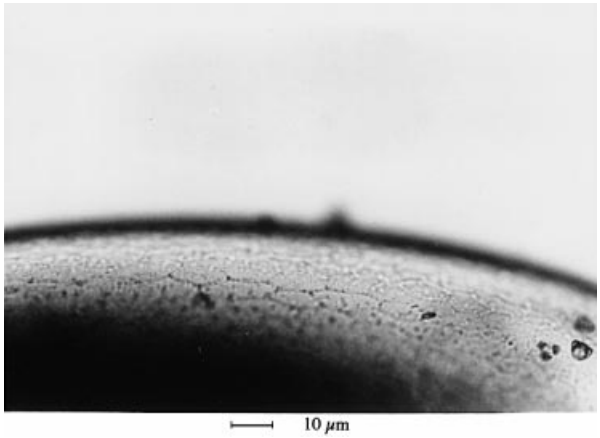

Fig. 3. Light microscopic detail of the outside of the eggshell of an intermediate patterned egg deposited by a *M. b. bicolor* worker. It reveals very weak patterning in the zone where, in the case of a patterned egg, there is an abrupt transition from the reticulate pattern to the smooth apex at the posterior pole.

Viability of worker-deposited eggs

In all five cases of cells oviposited in by the queen and closed by a worker in a normal way, the queen's egg produced a larva. A normal larva was found in three of the five cases where a cell which was oviposited in by the queen but was artificially closed by a small piece of wax. The two remaining cells were opened by workers and emptied, which shows that the artificial closing of cells may provoke workers to open

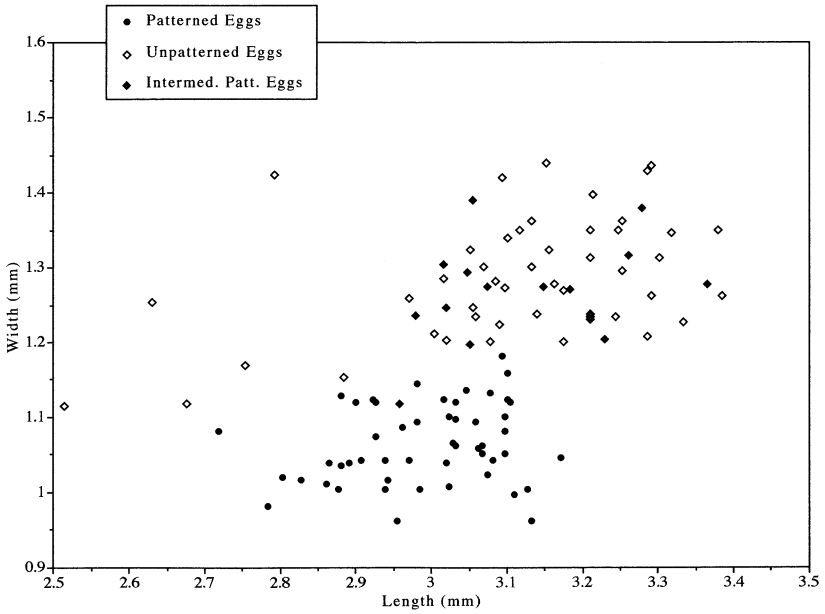

Fig. 4. Graphic representation of sizes of eggs (in mm) laid by workers of *M. b. bicolor*. The different symbols represent the different types of morphological categories with regard to the presence or absence of the reticulate chorion pattern: patterned, unpatterned and intermediate patterned.

them. Tests on the viability of worker eggs revealed that, of seven eggs laid by workers that started to close the cell immediately thereafter, six developed into larvae. One cell was re-opened by the workers. In 15 cases when we prevented the queen from eating the worker egg by covering the cell with a piece of wax, 10 cells were opened by workers and thereupon emptied. Five cells produced a larva. Three of these larvae were small when compared with typical larvae of that age.

Discussion

Worker egg laying, egg morphology and viability

In the majority of the cases, the relation between the behaviour of the laying workers in *M. b. bicolor* and the morphology of their egg was obvious. The relation between workers laying an egg after oviposition by the queen and worker egg morphology seemed to be unequivocal; the worker always closed the cell after egg laying and her egg was always patterned. Also, under queenless conditions, relatively more patterned eggs and eggs with intermediate chorion patterning were laid than eggs that were unpatterned. However, our results demonstrate that in *M. b. bicolor* other configurations in the relation between the behaviour of laying workers and the morphology of their egg also exist. The situation found in *M. b. bicolor* differs from *M. favosa*, where there seems to be a strict connection between the moment of egg laying by a worker, the type of egg produced and subsequent worker behaviour (Sommeijer & van Buren, 1992).

In *M. b. bicolor*, unpatterned eggs were clearly bigger than patterned eggs but less so than in, for example, *T. angustula* (Koedam *et al*., 1996). In many species of *Trigona*, workers lay trophic eggs on the inner side of the upper cell margin to be eaten by the queen (Sakagami, 1982). In *Melipona* the differences between worker egg types and the way in which they are deposited are, in general, not as distinctive as in *Trigona*, which may also be because in the former both are laid at the same location in the cell, namely in the centre of the cell on the surface of the provisioned food. This probably led some authors to state that in *Melipona* the laying of only one type of worker egg occurred (Bego, 1983; Inoue *et al*., 1999).

In the supposed conflict between queen and workers over male production in social insects (Hamilton, 1964), the consumption of eggs by nestmates should, following the usual line of thought, lead to more refined ways by which egg-laying workers avoid their reproductive eggs being eaten. In many species, workers succeed in producing males (Beig, 1972; Contel & Kerr, 1976; Machado *et al*., 1984; Bego, 1990; Koedam *et al*., 1999; Sommeijer *et al*., 1999), so this route in behavioural and physiological development has been taken often. On the other hand, continual egg consumption by the queen or by sister workers makes the chances for worker-laid eggs ever to hatch low. This, consequently, should have selected for workers to invest less in the making of a full-grown, viable egg and to take less care where and when to deposit the egg. *Plebeia remota* is a species in which the moment and place of deposition of worker eggs is no longer of prime importance; the majority of worker ovipositions occur in the phase characterized by the cruising of the queen along the row of newly constructed cells up to moment the first food discharges occur (van Benthem *et al*., 1995; see also Zucchi, 1993). That the viability of the egg is then no longer of prime importance is clearly shown by the fact that in *M. b. bicolor* some of the eggs laid by the workers quitting the cell, thereby giving the queen the opportunity to eat them, were less viable than others. According to the retarded development of the larvae, in some cases the behaviour of these workers already reflects a first step towards a decline in egg viability.

The regular presence of worker eggs serving as food in many stingless bee species, as well as eggs serving a reproductive function, and their corresponding variation in size and morphology, indicate the existence of selection pressures leading to similar physiological developmental routes in the different taxa, as well as to a collective origin in their evolution. The occurrence of a dichotomy in the developmental programme of eggs, which can be noticed even from the performance of a single worker displaying one type of egg laying within a few days of another type (Koedam *et al*., 1999), probably reflects the simultaneous presence of altruistic behaviour, serving the benefit of the colony, and selfish elements of worker fitness.

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