**Research article** 

# Clustered male production by workers in the stingless bee *Melipona subnitida* Ducke (Apidae, Meliponinae)

# D. Koedam, F.A.L. Contrera and V.L. Imperatriz-Fonseca

Laboratório de Abelhas, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, Travessa 14, No. 321, CEP 0558-900, São Paulo, SP, Brazil, e-mail: dkoedam@usp.br

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Summary. In stingless bees brood cells are sequentially filled with liquid larval food (mass-provisioning), upon which the queen lays an egg. Thereafter the cell is closed by a worker. This study showed that during these processes workers of Melipona subnitida regularly laid eggs that served as food for the queen. Occasionally cells were oviposited in and immediately closed by a worker. These cells always rendered males. Some of these reproductive workers were seen to lay a trophic egg as well. Cells which were exclusively oviposited in by the physogastric queen gave rise to workers and queens only. In one colony it could be verified that three workers alone, which differed in age by one day, laid 15 male-producing eggs within a period of two successive weeks. Among them the number of ovipositions was positively related to the order in which workers eclosed - the oldest worker laying most eggs - and inversely related to the number of times they closed cells oviposited in exclusively by the queen. Apparently the physogastric queen was not able to stop certain workers from reproducing. We therefore conclude that some workers in M. subnitida temporarily dominated their queens in egg-laying.

*Key words:* Stingless bees, *Melipona subnitida*, worker reproduction, male production, queen dominance.

## Introduction

In stingless bees a brood cell is provisioned by workers with liquid food. The queen then lays an egg on top of this food, and the cell is closed by a worker. Unlike honey bees and other stingless bees, in the genus *Melipona*, bees of both sexes and castes emerge from similar sized cells (Sakagami, 1982). Like the other Hymenoptera, stingless bees are haplodiploid, which means that females are able to control the sex of their offspring: fertilised eggs generate females, unfertilised eggs give rise to males. In many species of stingless bees workers lay eggs during the provisioning and ovipositioning of cells, in general immediately after a series of regurgitations (Sakagami, 1982). When these worker-laid eggs are eaten by the queen, they are called trophic. For several species it has been demonstrated that trophic eggs are distinct from reproductive eggs in morphology and/or quality (Akahira et al., 1970; Cruz-Landim and Cruz Höfling, 1971; Sommeijer et al., 1984; Koedam et al., 1996). Other worker eggs hatch and give rise to male offspring. In these cases, a laying worker immediately seals the cell orifice after her ovipositioning.

In *Melipona subnitida, M. favosa* and *Plebeia droryana* workers contribute in a considerable way to male production (Contel and Kerr, 1976; Machado et al., 1984; Sommeijer et al., 1999). Thanks to the variable origins of males and the difficulty of determining the moment at which they are produced (Bego, 1982, 1990), the mechanism for the production of males in stingless bees is still unknown.

In some stingless bee species workers commonly lay reproductive eggs when their colony is queenless (Imperatriz-Fonseca and Oliveira, 1976; Sakagami, 1982; Sommeijer et al., 1984), which shows that the queen's presence suppresses worker reproduction. It is generally believed that in large colonies of social insects, queen control over reproduction by workers and supernumerary queens is maintained by a ritualised dominance behaviour in which pheromones play a more important role, rather than direct aggression (see Keller and Nonacs, 1993).

Production of males can lower colony fitness in two ways. First, reproductive workers might behave selfishly, and therefore perform badly in other nest tasks (see Montague and Oldroyd, 1998). Secondly, male production might occur at the expense of worker production (Koedam et al., unpublished data). So, the cost and benefits of male production, especially by workers, should constantly be weighted against colony interest. In the present study we investigated the production of males in queenright colonies of *M. subnitida* by means of behavioural observations and caste- and sex-ratio analyses of brood combs. We searched for possible patterns in male production and we tried to discover which of the female castes produces males.

## Materials and methods

To study male production we used four colonies of *Melipona subnitida* collected at Jardim do Seridó, Rio Grande do Norte, Brazil. The bees were studied in the Bee Laboratory at the University of São Paulo. On 9 January, 1997, before the colonies were transported to São Paulo, we removed the physogastric queens. The colonies selected one of their gynes which could fly out and be inseminated, and then develop into the physogastric queens were marked with paint on the dorsal part of their thorax and the colonies were transferred into wooden observation hives. The hives were placed in a dark room with a controlled temperature of a bout 28 °C. The hives were connected to the outside by means of a plastic tube, allowing the bees to forage normally. The colonies had populations of about 400–500 bees each.

To identify workers and to follow them in their activities, recently emerged workers were marked dorsally on their thorax with coded paper tags. First, the oldest combs were isolated from the nest and were placed into a small wooden square dish which was connected to the main hive by means of a plastic tube. The bees were able to pass through this tube in both directions. Recently emerged workers were distinguishable from adult bees by their pale coloured exoskeleton. While their exoskeleton hardened, workers remained inactive for several days, which made it possible to collect and mark them at intervals of 24 hours. After marking, the bees were placed back into the main hive. The marking of individuals in all four colonies was started on the 17th of April and finished on the 25th of July.

Provisioning and ovipositioning processes (POPs) were observed in four colonies up to three months by means of direct observations. At night POP-behaviour was registered with the aid of video cameras. By making notes of the precise position of cells in the combs, we were able to relate the behavioural events recorded at the cell to the sex and caste of the individual that would emerge from it.

Cells containing pupae were opened to determine the sex of the individuals before emergence. A comb was taken from a colony 36-37 days from the date the cells in its center were oviposited in. After we carefully removed the caps of the cells, sex could be determined using the shape and relative size of the head and eyes of the pupae (Camargo et al., 1967). When the face of the pupa had been damaged during the procedure of opening, the pupa was taken out of the cell and the caste/sex was determined with the help of the overall body shape. Males eclosing from cells in which both the queen and a reproductive worker oviposited are considered to be worker-derived (Beig, 1972). After having opened all the cells of a comb, the cells were closed with wax parts taken from the nest. Once the comb was placed back into the hive the bees could emerge normally. From each of three colonies four consecutively constructed combs were taken to determine the caste- and sex-ratios. For the same purpose we collected two consecutively constructed combs from a fourth colony.

For a study of the distribution of male cells in combs, combs were divided into three sections according to the dates on which the cells had received eggs. Each section contained equal numbers of cells. Because in general combs were disk-shaped and because oviposition in cells occurred from the center outward, the three sections turned out to be concentric: central, middle, and peripheral. Next, the proportion of male cells per section was determined.

### Results

#### Trophic and reproductive egg-laying by workers

The 14 monitored combs contained a total of 2496 cells, in 891 of which we saw oviposition. Trophic egg-laying was observed in all four colonies. Workers laid these eggs at an average age of 15 days (mean = 15.1, SD = 2.6, n = 170, Fig. 1). Occasionally, workers laid reproductive eggs and this was observed in three of the four colonies. The average age at which workers laid these eggs was 15 days (mean = 14.9, SD = 3.4, n = 16, Fig. 1). Workers laying reproductive eggs started at a younger age than workers laying trophic eggs (age-classes (in days): <10, 10–12, 13–15, 16–18, 19–21, >21; chi-square 19.08, p = 0.002, Table 1). The closing of cells was performed by bees having an average age of 9 days (mean = 9.2, SD = 2.6, n = 527, Fig. 1).

From the caste- and sex-ratio analysis of the 14 combs, we found 30 cells which contained male pupae. Of these, 16 cells were observed to be oviposited in by a worker. Of the remaining 14 cells the ovipositioning had not been registered.

After the study of this set of combs additional observations revealed that a male-determined egg was produced in colony 1 and that it originated from a worker. This single observation brings the number of males produced in our *M. subnitida* colonies up to a total of 31.

On eleven occasions reproductive workers produced their egg directly after the series of regurgitations which prevented the queen from ovipositing. In all cases, the queen physically tried to hinder the worker from closing the cell by drumming violently on her. This aggression faded away only after the worker had the cell completely sealed.

On six occasions a reproductive worker laid her egg right after the oviposition by the queen or during the phase of cell closing. These cells received two eggs: one from the queen



Figure 1. The number of *M. subnitida* workers which, 1. laid reproductive eggs, 2. closed cells, and 3. laid trophic eggs, in relation to their age. Note that the behaviour "closing cells" does not include bees that closed a cell directly after they had laid a reproductive egg

Worker Eggs		Age-cla	Age-classes in Days									
		<10	10-12	13-15	16-18	19-21	>21					
Reproductive	observed	1	3	4	5	3	0					
	expected	0.09	1.72	8.09	4.90	0.95	0.26					
Trophic	observed	0	17	90	52	8	3					
	expected	0.91	18.28	85.91	52.10	10.05	2.74					

Chi-square = 19.08, *P* = 0.002.

and one from the worker. Since the queen was in the habit of walking away from the cell following egg-laying, workers laying reproductive eggs after the queen's oviposition in general closed the cell without being violently pummeled by the queen.

## Individual behaviour of reproductive workers

The three workers that were responsible for a considerable part of the male output in the two-week cluster in colony 3 differed only one day in age: they had emerged on the 3rd, the 4th and the 5th of May 1997, respectively (from this point on these workers are referred to as A, B and C resp.). Worker A laid 10 reproductive eggs, worker B laid four and worker C produced only one (Fig. 2). Like non-reproductive workers, they were observed operculating cells which were oviposited in exclusively by the queen. The frequency with which they performed this cell operculating was inversely related to the number of eggs the individual workers produced (Fig. 2).

During a period of three weeks, worker A modified her reproductive behaviour in relation to the moment she laid her egg in POP; she produced her first egg on the 14th of May just after a series of regurgitations and before the queen could lay an egg. Two days later she produced her next egg, which was laid after the queen had oviposited. Six days later she performed another egg-laying sequence by pushing aside a non-reproductive that was closing a cell in a regular way, immediately laving her egg and closing the cell. When, on another occasion but in a similar situation, worker A pushed worker B from a cell, a short but intense fight occurred between them. Just before the moment of attack, worker B had been engaged in sealing this cell but she had not oviposited in it, although she was active in reproduction around that time. During the fight they fell off the upper comb. A few seconds later, worker A again arrived at the abandoned cell, rapidly laid her egg and closed the cell. Five and six days after she laid her last reproductive egg, on the 28th and the

 
Table 1. A comparison of the distributions of reproductive worker eggs and trophic eggs with respect to worker-age in *M. subnitida*

29th of May, worker A was seen to lay a trophic egg (Fig. 2). She was then aged 25 and 26 days, respectively. In all, four workers that produced reproductive eggs were observed to produce trophic eggs as well.

## Clustered male productions

Over a period of two consecutive weeks 23 males were produced in colony 3 and in 15 of these cases we confirmed that three workers alone were responsible for this output (Table 2, Fig. 2). In colony 2 the production of six males occurred (Table 2). From this series, we only observed the laying of the first male-determined egg and it was laid by a worker. The two clusters of males were produced in eight days and in two weeks respectively (Table 2).



**Figure 2.** (a) A schematic representation of the individual development over time of the three reproductive workers A, B and C regarding egglaying as it was observed in *M. subnitida* colony 3. The letters R and T stand for the laying of reproductive and trophic eggs. The numbers adjacent to these letters reveal whether the egg was laid immediately after the series of regurgitations, thereby excluding the queen from ovipositing in that cell (1), whether it was laid following the queen's oviposition (2) or whether it was laid by pushing away the worker which was already closing the cell (3). The total number of male-determined eggs produced in the period under consideration is depicted in Table 2. (b) The number of times these workers closed cells which were oviposited in exclusively by the queen

**Table 2.** A representation of two clustered production series of males over time in *M. subnitida*. In the series that occurred in colony 3 we could verify that at least 15 of a total of 23 males were worker-derived (see also Figure 2). In the other series we could only observe the laying of the first haploid egg and this was performed by a worker

Day	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th	16th
Colony 2 Colony 3	1 1	_	_	_	1 1	1 1	2 3	1 2	-3	$\frac{-}{3}$	-3	$\frac{-}{2}$	-3	_ 1	_	_

Colony	Comb Section	Proportion	of Male Cells	No. Cells/Comb		
		Central	Middle	Peripheral		
2		0.031	0.078	0.008	158	
3		0.021	0.000	0.000	159	
3		0.012	0.003	0.031	210	
3		0.119	0.100	0.004	273	
	Average	0.046	0.045	0.011		
	SD	0.049	0.051	0.014		

Proportions represent the number of male cells per section to the total number of cells per section.

**Table 3.** The distribution of male cells in combs of *M. subnitida*. Combs were divided into three sections according to the dates on which the cells had received eggs. Each section contained equal numbers of cells. Because in general combs were disk-shaped and because oviposition in cells occurred from the center outward, the three sections turned out to be concentric: central, middle, and peripheral

Just as the workers produced their male-producing eggs concentrated in time, cells containing male-pupae were aggregated in combs. In addition to this, the male-pupae holding cells were predominantly located in and near the center of the combs (Table 3).

## Production of sex and caste

Four combs from colony 1 comprised a total of 641 cells: 583 workers and 50 queens. Four combs from colony 2 consisted of 669 cells: 608 workers, 34 queens and 6 males. Four combs from colony 3 were composed of 857 cells: 726 workers, 65 queens and 24 males, and two combs from colony 4 had only workers and queens, 298 and 19 respectively. Mainly because of the necessary manipulation with the combs, 3 per cent of all cells was lost.

## Discussion

#### Clustered male production

The clustered productions of males as recorded for *M. subnitida* is similar in appearance to the clusters which have been seen during four months' research in three colonies of *M. favosa* (Koedam, unpubl. data). This study reveals that such a series of males can be the result of egg-laying by several workers concentrated in time. We did not notice that the queen produced males. Yet, a more recent study on *M. subnitida* revealed that the queen can share considerably in the production of males (Koedam et al. unpubl. data).

As in the study of *M. favosa* (Koedam, unpubl. data), in *M. subnitida* cells having male pupae were found in or near the center of combs. Kerr (1950) stated that in combs of various species of *Melipona* "drone cells were found chiefly at the center". Why the laying of male-producing eggs, and possibly the appearance of reproductive workers, occur when a comb is nearly completed and workers focus their building activity towards the first cells of the next new layer of brood cells is still an unsolved matter.

## Physiology of worker egg-laying

Since trophic eggs constitute an important part of the nourishment of physogastric queens (Velthuis, 1993), selection will have favoured workers to lay eggs in this manner. The force of selection for this type of behaviour by means of a disconnection of the behaviours "egg-laying" and "cellclosing" is in particular demonstrated by the generally continuous presence of many non-reproductive workers which close cells and lay trophic eggs during a narrow age window.

In Nannotrigona (Scaptotrigona) postica reproductive eggs are produced predominantly by young workers whereas trophic eggs are produced mainly by relatively older ones (Bego et al., 1983). In *M. subnitida* we found that the average age at which workers laid reproductive and trophic eggs is approximately the same. However, reproductive *M. subnitida* workers seem to start laying eggs early in life compared to workers laid trophic eggs. Also, when a *M. subnitida* worker laid both reproductive and trophic eggs, she always laid the latter type after her reproductive phase.

## Queen dominance?

Our study shows that in *M. subnitida* certain workers were capable of pursuing a reproductive strategy in which they temporarily dominated the queen in egg-laying. This hindering of the queen in her ovipositioning can be considered as a selfish act. The inverse relation between the number of eggs a reproductive worker laid and the number of times she closed cells which were oviposited in exclusively by the queen accentuates the selfishness of worker reproductivity.

Koedam et al. (1997) showed that in colonies of *Trigona* (*Tetragonisca*) angustula the queen by ovipositing prevented workers from laying the maximum possible number of trophic eggs. This study shows that at times the queen tried to hinder the reproductive workers in ovipositing cells but she never visibly restricted them laying their eggs. Obviously in *Melipona* a queen is not able to beat a worker behaviourally because the queen and workers are less differentiated in size and the queen's morphology is adapted exclusively to egglaying. Given that workers in *M. subnitida* reproduce unhindered, the question is raised of how and when the phero-

monal influence of the queen, which is supposed to play an important role in ritualised dominance in highly social insects, operates on workers.

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