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Research article

How queen and workers share in male production in the stingless bee *Melipona subnitida* Ducke (Apidae, Meliponini)

D. Koedam, F. A. L. Contrera, A. de O. Fidalgo and V. L. Imperatriz-Fonseca

Laboratório de Abelhas, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, Travessa 14, No. 321, CEP 05508-900, São Paulo, SP, Brazil, e-mail: dkoedam@usp.br

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Summary. Potential conflict between the queen and workers over the production of males is expected in stingless bees as a result of the higher relatedness of workers with their sons than with their brothers. This conflict was studied in *Melipona subnitida* by observing how the queen and the workers share in male production. The oviposition of individual cells was observed in two colonies with individually marked workers for a period of 51 and 40 days respectively. The gender that developed from these cells was then determined. The results revealed that most male production was concentrated in a 2 – 3-week period, during which laying workers were present. During these weeks, the queens produced twice as many males as all laying workers together. Outside this distinct period, the queens produced an occasional male. A reproductive worker either oviposited before the queen did, in which case she immediately proceeded to close the cell and thus prevented the queen from oviposition, or oviposited and sealed the cell after the queen had laid an egg. When cell construction and oviposition occured on several combs simultaneously, the workers preferentially laid male eggs on the newest combs. We discuss the proximate mechanism and ultimate cause of the way in which queen-worker male production occurred. In conclusion, we argue that overt behavioural conflict, occasionally displayed by reproductive workers of this species, can be of great cost to the colony.

Key words: Stingless bees, *Melipona subnitida*, reproductive workers, male production, queen-worker conflict.

Introduction

Highly eusocial stingless bees live in tropical and subtropical parts of the world and their perennial colonies comprise a few hundred up to several thousand individuals, mostly females (see Michener, 1974; Sakagami, 1982). Normally, one queen lays the eggs and lives together with her daughters, who take care of the brood, protect the nest, and forage for food. The long-lived queen in the genus *Trigona* is truly bigger than the short-lived daughter-workers, while this size difference is only reflected in the queen's inflated abdomen in *Melipona*; in fact, *Melipona* queens are somewhat smaller than workers at eclosion even though both castes originate from similar-sized brood cells. The nests also generally contain up to a few young, uninseminated queens although in *Melipona* they are normally killed soon after emerging. Callow workers start their life working on the brood and, with age, move to other parts of the nest where they engage in other activities until they start foraging. Males leave the nest a few days after eclosion.

Offspring are produced in brood cells that are often arranged in horizontal combs. These brood cells differ clearly in size from the food-storage pots (Wille and Michener, 1973). In these species, new cells are constructed along the periphery of the newest comb, which is started on top of the previous comb shortly before it reaches its final size. This pattern of building combs one on top of the other generates a vertical column of combs in which a relatively young brood is situated above an older brood, as long as space permits. After the bees have emerged, the old cells are dismantled (Sakagami, 1982). All stingless bees show mass provisioning of brood cells; several workers first regurgitate liquid food for future larva in a cell. When the cell is filled with a sufficient amount of food, an egg is laid in it, normally by the queen. Then, the cell remains closed until the nearly full-grown bee emerges. The common shorthand term for this process of brood-cell provisioning and oviposition is POP (Sakagami, 1982).

Colonies of stingless bees are generally monogynous; however, reproduction by workers is common (Beig, 1972; Machado et al., 1984; Beig et al., 1985; Bego 1990; Koedam et al., 1999; Sommeijer et al., 1999; Tóth et al., 2002b). Though uninseminated, the haplo-diploid system of sex determination allows workers to produce male offspring

(Beig, 1972; Zucchi, 1993). In addition to these reproductive eggs, workers produce trophic eggs. This phenomenon is widespread among stingless bees (Sakagami, 1982; Zucchi, 1993). In general, it is during POP that workers lay their eggs (Sakagami, 1982).

Trophic eggs are supposedly sterile or immature (Akahira et al., 1970; Cruz-Landim and Cruz-Höfling, 1971) and are usually consumed by the queen. These eggs are bigger, have a slightly different morphology, especially their chorion structure (Sommeijer et al., 1984; Koedam et al., 1996; 2001), and, in a number of species, are laid in another spot than the reproductive eggs, such as at the cell rim or on the comb surface (Sakagami and Zucchi, 1966). In the genus *Melipona*, however, both trophic and reproductive eggs are laid on food in the centre of the cell. Behavioural differences are also seen in the workers with regard to laying a trophic or a reproductive egg (Bego, 1990; Sommeijer and van Buren, 1992; Koedam et al., 1999). After the workers have provisioned a new cell in the presence of the queen, one worker may position herself on the cell to lay an egg. When she leaves the cell, the queen eats the worker's egg. If the worker does not leave, she will start closing the cell immediately after laying, thereby preventing the queen from laying an egg. The queen may beat the worker's body with her antennae and forelegs while the worker lays an egg. Eventually, the reproductive worker seals the cell completely. A worker may also lay a reproductive egg after the queen oviposits, resulting in a cell with two eggs.

The kin selection theory predicts a potential conflict between the queen and the workers with regard to the production of males. This is caused by the higher relatedness of workers with their sons than with their brothers (Hamilton, 1964). The structure of this conflict is thought to be different under single and multiple mating: workers of a multiply-mated queen should police each other to favor the production of brothers, whereas workers of a singly-mated queen should collectively oppose their mother in her male production (Ratnieks, 1988; Queller and Strassmann, 1998). Since stingless bee workers are usually daughters of a single, singly-mated queen, they are expected to produce the males (Peters et al., 1999). However, the actual outcome of the conflict over male production in stingless bees varies and is supposed to depend on the control over who reproduces, the costs of male production by workers, and phylogenetic constraints (Tóth et al. 2002a,b; 2004).

The queen and the workers of *M. subnitida* produce males in a ratio of about 3:2 (Contel and Kerr, 1976), although it is still not clear how this sharing is achieved. Observations by Koedam et al. (1999) showed that some workers may temporarily control the egg-laying process. In cases of reproductive worker egg laying, the queen was never able to push laying workers from the cell nor was she seen to re-open a cell oviposited in by a worker and/or replace the worker's male egg with one of her own. For this paper, we studied the conflict regarding male production in *Melipona subnitida* by examining how the queen and the workers share in male production. The observations on egg-laying behaviour and the analysis of the resulting sex and caste revealed that the two castes produced males simultaneously. We discuss the regulatory role of food in male egg laying and the outcome of the queen-worker conflict over male production as we found it. We have reasons to believe that the occasional occurrence of aggressive behaviour presented by workers laying male eggs could involve considerable costs to the colony.

Materials and methods

Monitoring colonies for male production

This study was carried out at the Bee Laboratory, University of São Paulo, Brazil, during March and April 1998. Two free-foraging *Melipona subnitida* colonies of medium size (400–500 bees) were studied with regard to male production; each colony contained a physogastric queen. The queens were originally marked with paint on their thorax in the beginning of 1997. The provisioning of and ovipositing in cells by individually marked bees was registered daily by means of direct observations. To make identification possible, emerging workers were marked on a daily basis with coded paper tags glued on the thorax (see Koedam et al., 1999 for the technique of marking individuals). The marking of bees was started on 27 February 1998 in colony 1 and on 9 March 1998 in colony 2.

Monitoring started on 8 March 1998 in colony 1 and on 19 March 1998 in colony 2, and continued until 28 April 1998. The colonies produced a total of 1860 cells (colony 1: 1001 cells in 51 days; colony 2: 859 cells in 40 days), the complete sequence of provisioning and ovipositing was recorded in 1069 cells. Of the 1860 cells, 232 cells were re-opened later by workers and emptied (colony 1: 129 cells; colony 2: 103 cells), so information about the sex and caste of the offspring they contained was thereby lost. Of these lost cells, seventeen had received a reproductive worker egg.

Cell destruction mostly occurred because workers actively opened the cells and removed the contents. A small number of closed cells was lost due to damage during manipulation of the combs. As a result of small experimental manipulations not related to this study, seven cells were re-oviposited in by the queen or by a worker.

Knowing the sex and caste of the bees that emerged from the cells in which ovipositing was observed makes it possible to determine the proportion of males produced by each of the castes. To do this, each comb, of which the cells and the origin of the eggs were mapped, was taken from the colony 35 days after its first cell was produced. During this time interval, the larvae had pupated and the heads of the bees were already sufficiently differentiated to recognize them as being a queen, a worker, or a male. For this identification, the cell lids were carefully removed. After having noted the caste and sex of the pupae, the cells were closed with pieces of wax taken from the nest. The comb was then returned to the colony, where a normal hatching of the individuals followed. Within an hour after these manipulations, workers resumed their normal brood tasks. More information about this non-invasive sampling method can be found in Koedam (2003).

Initiating male production

Because reproduction by workers probably depends on the amount of available proteins (Koedam, 1999), a surplus of pollen with a small quantity of honey originating from a *Scaptotrigona postica* nest was placed inside each of the two observation hives on 2 April 1998. The laying of male eggs by the worker caste occurred from 5 April to 25 April in colony 1 and from 15 April to 28 April in colony 2, when we ceased observations. During the periods of worker reproduction, ovipositing was monitored 24 hours a day by means of direct observations. For 20 days and 17 hours, a continuous series of 462 POPs on four consecutively constructed combs (*c*, *d*, *e*, and *f*, see Fig. 3) was recorded in colony 1.

Ovipositing in seven cells of colony 1 escaped our attention. For 12 days and 22 hours, a continuous series of 327 POPs on three consecutively constructed combs (*d*, *e* and *f*, see Fig. 3) was recorded in colony 2. Ovipositing in two cells of this colony escaped our observation.

Analysis of male production

We looked for patterns in the simultaneous production of males by the queen and the workers. First, the sequence in which they oviposited cells was analysed. That is, the order in which cells received a queen's egg or a worker's egg and the order in which they received the queen's male egg or a worker's male egg were examined with a Runs test (Siegel and Castellan, 1988). A Change-point test (Siegel and Castellan, 1988) was applied for the individual combs, to see whether one of the castes was earlier in laying male eggs than the other. Only combs *d* and *e* of colony 1 and comb *e* of colony 2 were analysed, because the production of males in the other combs by both castes was very small or slowly ceased during construction or because observations were stopped.

Comb preference in the laying of male-determined eggs was analysed with the help of a Chi-square test. The same test was used to see whether there was a relationship between workers interrupting their series of male egg-laying on one comb to continue on another and their behaviour on a previous egg-laying occasion or on a following egg-laying occasion and whether this worker oviposited directly after a series of regurgitations, thereby preventing the queen from laying an egg, or whether the worker oviposited after the queen's egg laying in the same cell, resulting in a cell having two eggs.

The results of the statistical analyses of male egg laying refer to the situation in which males from cells in which both the queen and a worker oviposited are assumed to be worker-derived. Performing statistical analyses under the assumption that cells in which both the queen and a worker oviposited give rise to males derived from the queen only, did not change the outcomes significantly.

P values (two-tailed) for significant outcomes are quoted as an exact probability value and non-significant outcomes are indicated as NS. When using chi-square analysis, Yates's correction for continuity was applied.

Results

Reproductive egg laying by workers

Workers laid a total of 104 reproductive eggs, 101 of which were produced by 24 individuals. Three eggs were laid by unidentifiable workers. In both colonies, the various reproductive workers contributed differently in the laying of reproductive eggs. Twelve of the 24 workers laid only one or two eggs, while the rest laid between three and eleven eggs each. Workers often laid their eggs subsequently on separate days, with intervals of up to five days. For colony 1, worker egg laying started on 5 April, reached the highest peak on 13 April, and ended 12 days later. The frequency distribution of the daily egg layings by reproductive workers in colony 2 is similar to that found for colony 1. This series of worker egg layings commenced on 15 April, showed the highest incidence on 20 April, and continued until the last day of observation, seven days later.

A reproductive worker laid an egg directly after a series of regurgitations 68 times. After ovipositing, the worker stayed on the cell and immediately began to close the cell by folding the cell rim inwards, thereby preventing the queen from laying her egg. The queen, waiting nearby, always vigorously tapped the worker with her antennae and forelegs (Fig. 1). In 34 cases, the worker laid an egg after the queen oviposited. This resulted in a cell with two eggs because the worker did not eliminate the queen's egg prior to laying her own. In two remaining cases, information about the timing of worker egg laying was lost.

The queen always immediately walked away from the cell after oviposition, but did not leave the combs. Her presence on the comb occasionally resulted in a brief interaction between herself and the worker sealing the cell. This worker, however, was never vigorously drummed, even when she had laid an egg following the queen's oviposition. The queen was never observed to open a cell containing a worker's egg and replace it with one of her own.

In general, reproductive workers were seen to oviposit in cells that had not yet been oviposited in by other workers. On one occasion, we saw workers opening a cell that had been oviposited in previously by a reproductive worker. The cell was emptied completely and, six hours later, in the presence of the queen, filled again with liquid food upon which another reproductive worker laid an egg.

One act of aggression by a reproductive worker directed towards the queen was observed. While the queen was positioning herself on the cell, a reproductive worker grasped the queen's mandibles and pulled her from the cell. Immediately afterwards, the worker mounted the cell, laid an egg, and sealed the cell. As in every case where a worker prevented the queen from ovipositing, the queen tapped this worker vigorously during oviposition and cell sealing. Aggressive interaction among reproductive workers was not observed.

Figure 1. Using her antennae and forelegs, the queen taps a tagged worker that has just laid a male-producing egg and is closing the cell

Queen sharing in male production

During the entire period of observation, the brood comprised 22.7% males in colony 1 (n = 198 males) and 10.8% males in colony 2 (n = 82 males; Table 1). Male production was concentrated in a 2–3-week period during which laying workers were present. Outside these weeks, the queens irregularly laid male eggs (n = 11 males, Table 1). The queens produced about twice as many males as all the laying workers together (colony 1: 136:53; colony 2: 50:30). We were not able to determine the origin of 11 males. Under the assumption that cells in which both the queen and a worker had oviposited gave rise to a male from the queen alone, the queen-worker male production ratio changes from 2.6 to 4.6 in colony 1 and from 1.7 to 2.6 in colony 2 (Table 1).

The daily production of new cells in colony 1 and colony 2 was, respectively, 1.2 and 1.3 times higher during the period of worker reproduction (period B) than outside this period (period A). Compared to period A, the number of workers produced during period B was lower by 5.0 per day in colony 1 and higher by 1.2 per day in colony 2. As a result of the concentrated male production, the proportion of males rose from 2.9 to 47.2% in colony 1 and from 0.2 to 25.5% in colony 2 (Table 1).

Workers were seen to lay a total of 292 trophic eggs during 1231 POPs. The daily frequency of trophic egg laying in colony 1 was about 1.6 times higher in period B than in period A, whereas the number of trophic eggs per POP in period B was 1.2 times higher than in period A (period A:B; TWEs 71:89, POPs 356:368). The daily frequency of trophic egg laying in colony 2 was nearly equal for the two periods, while the number of trophic eggs per POP in period B decreased to 0.8 times that in period A (period A:B; TWEs 82:50; POPs 288:219). The queens generally ate the trophic eggs.

For as yet unknown reasons, workers in both colonies actively opened cells shortly after the introduction of surplus pollen. During the 2–3-week period of concentrated male production, 72 cells were destroyed by worker opening in colony 1 (Table 1). Most of these cells were from the older combs, i.e. *c* and *d* ($\chi_3^2 = 35.8$, $p < 0.001$), and they included cells oviposited in exclusively by the queen as well as cells oviposited in by reproductive workers (combs *c* and *d*: $\chi_1^2 = 0.21$, NS). In colony 2, nine cells were lost during period B.

Sequence of male egg laying

Patterns in the simultaneous production of male eggs by the queen and the workers were investigated. The queens started male production shortly after the workers (Change-point test: colony 1: $Z = -4.00$, $p < 0.001$, 7 April; colony 2: $Z = -3.86$, $p < 0.001$, 15 April): the queen in colony 1 started laying male eggs 68 POPs (about 67 h) after the first reproductive worker laid an egg. In colony 2, the queen started 35 POPs (about 32 h) after the first reproductive worker. In both colonies, the first reproductive worker's egg was laid directly after a series of regurgitations, thereby preventing the queen from ovipositing. During period B, queen and worker ovipositions occurred in random sequence (Runs test: colony 1: $m = 419$, $n = 60$, $r = 111$, $Z = 0.95$, NS; colony 2: $m = 301$, $n = 44$, $r = 82$, $Z = 0.91$, NS) as did the laying of male eggs by both castes (Runs test: colony 1: $m = 126$, $n = 53$, $r = 65$, $Z = -1.82$, NS; colony 2: $m = 49$, $n = 30$, $r = 40$, $Z = 0.31$, NS).

For individual combs, alternatives of whether the queen and the workers started the laying of male eggs at the same time or whether one of the castes was earlier than the other were examined. The results showed that, on one comb, workers were significantly earlier than the queen in laying male eggs (Change-point test: colony 1: comb *d*, $D_{58,7} = 0.719$, $p < 0.003$, Fig. 2). For the other combs, such an alternation in the laying of male eggs by either the queen or by the workers could not be detected (colony 1: comb *e*, $D_{62,37} = 0.245$, NS; colony 2: comb *e*, $D_{38,16} = 0.213$, NS).

Comb preference in male egg laying

The pattern of building combs one on top of the other implies that, at certain intervals, workers build simultaneously on two combs; i.e., some days, they construct the first cells of a new comb while finishing the previous one, thereby complet-

Table 1. The numbers of oviposited cells and the number of resulting offspring by sex and caste in the period with occasional male production by the queens (period A) and in the period with concentrated male production by both castes (period B) in the two colonies. The durations of period A and B in colony 1 were 30.3 and 20.7 days and in colony 2 27.1 and 12.9 days, respectively

Colony	1			2		
Period	A	B	B#	A	B	B#
No. Oviposited Cells	539	462		532	327	
Cells Lost	57	72		94	9	
Individuals Produced:						
Queens	43	18		34	29	
Workers	425	188		403	208	
Males	**14**	**184**		**1**	**81**	
Produced by:						
Queen	10	126	145	1	49	57
Workers	–	53	34	–	30	22
Unknown	4	5		–	2	

\# The numbers of males produced by the queen and the workers in period B under the assumption that cells in which both the queen and a worker oviposited gave rise to a male from the queen alone.

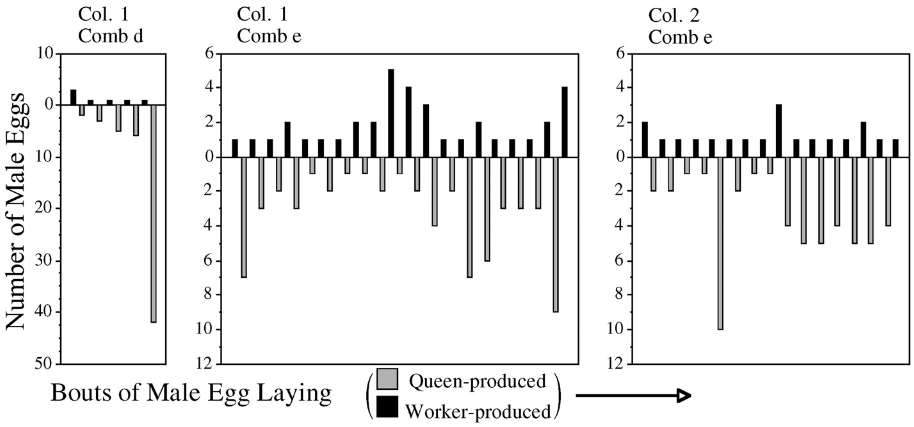

Figure 2. A presentation of the sequences in which the queen and the workers laid male eggs on three individual combs. Bars show the successive bouts of male egg laying by each of the castes over time, ordered from left to right

ing its final size (see Koedam, 1999 and this study Fig. 3). While doing this, cells are oviposited in according to the order in which they are being built on the different combs. The construction of three combs occurred simultaneously in colony 1 (Fig. 3).

A possible preference for the laying of female and male eggs was examined in cases where both the queen and the workers laid eggs on different combs simultaneously under construction. We found that the queen and the workers preferentially laid their male eggs in the newest comb. Figure 4 shows that this preference was clearly stronger for the workers than for the queens (data from colony 1 and colony 2 pooled, two combs: $\chi_2^2 = 18.5$, $p < 0.001$; three combs: $\chi_4^2 = 63.2$, $p < 0.001$).

In cases where two or three combs were simultaneously under construction, individual workers were seen to interrupt their series of male egg laying on one comb twenty times, only to continue on the next comb (n = 12 workers). Four workers did not switch combs. Of the comb switches, 15 were to the comb most recently under construction and one was from the comb in its final stage of construction to the comb in an advanced stage of construction. In three cases, switches were from the comb most recently under construction to the comb in an already more advanced stage of construction. In one case, the switch was from the comb in an already more advanced stage of construction to the comb in its final stage of construction.

We tried to determine whether a relationship existed between comb switching by workers and the queen being prevented from egg laying. Table 2 shows that when a worker arrived at the other comb to lay an egg, she was not found to be more inclined to lay the egg after a series of regurgitations, thereby excluding the queen, than to lay the egg after

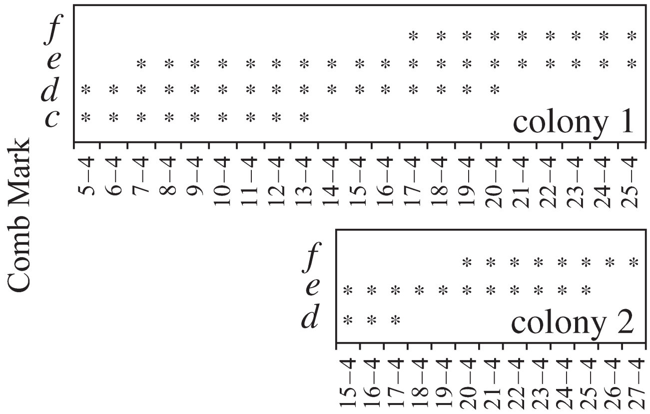

Figure 3. The days on which the construction of consecutive combs occurred during the 2–3-week period of concentrated male production (period B, April 1998)

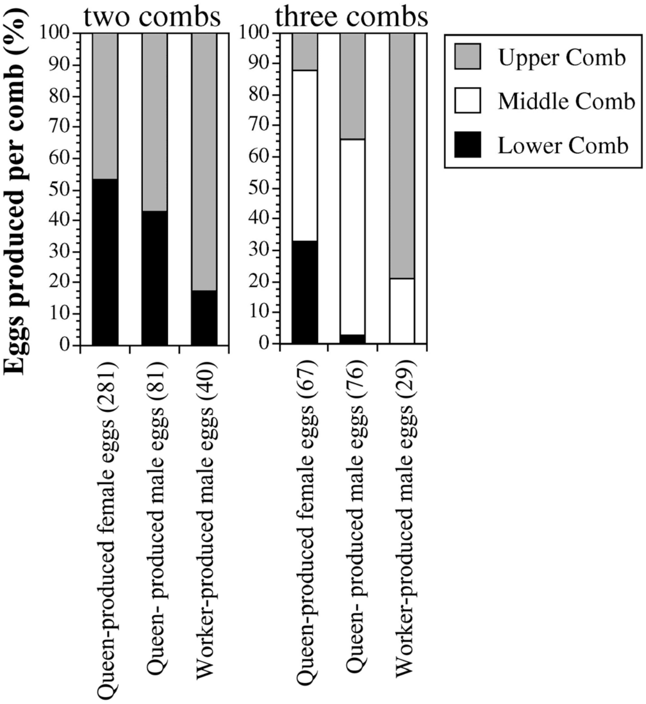

Figure 4. The production of female and male eggs by the queen and of male eggs by the workers on different combs simultaneously under construction. Between brackets: numbers produced, data from colonies 1 and 2 pooled

Table 2. The relation between the instant, during the provisioning and ovipositioning process (POP), at which one of two consecutive egg layings by individual reproductive workers occurred and the comb on which the other egg laying took place. A reproductive worker could lay an egg directly after a series of regurgitations, thereby preventing the queen from ovipositing in that cell, or after the queen's oviposition in the same cell. These two instants of egg laying by workers during POP were related to **(a)** the comb on which this worker would lay her next egg, and **(b)** the comb on which this worker had laid her previous egg. Data from the two *M. subnitida* colonies pooled

Comb	Preventing queen from ovipositing	After queen's oviposition
a. Next egg laying of reproductive worker		
Same	37	18
Other	16	4
b. Previous egg laying of reproductive worker		
Same	21	19
Other	17	3

the queen's oviposition in the same cell ($\chi_1^2 = 0.6$, NS). However, when a worker left the comb to lay an egg on another comb, she was likely to have excluded the queen during her previous egg laying ($\chi_1^2 = 4.75$, $p = 0.029$).

Discussion

Food conditions and male production

Food reserves have a positive effect on the production of sexuals (Veen et al., 1992; Moo-Valle et al., 2001) and, in the current study, we could stimulate both the workers and the queen of two *M. subnitida* colonies to lay male eggs simultaneously by supplying them with a large amount of pollen inside the nest. The different numbers of males that each colony rendered may have been due to small colony differences like size or queen physiology. Also, because the period of observations was relatively short, a natural asynchrony among colonies in the production of males by workers (Sommeijer et al., 2003) may have enhanced these different male figures. Nevertheless, in the current study, the queens were the main cause of the colonies' differences in male production (Table 1).

Male production by workers in *M. favosa* and *M. subnitida* (Koedam, 1999; Koedam et al., 1999; Sommeijer et al., 2003; Chinh et al., 2003; this paper) occurs in bouts of varying size and several hypotheses have been presented to explain this occurrence (Chinh et al., 2003). Like in a former study on *M. subnitida* (Koedam et al., 1999), the present study reveals that time-clustered male egg layings can result from the joint, but differential contribution of various individual workers that lay their eggs over a short period of consecutive days or weeks (see also Koedam and Imperatriz-Fonseca, in prep.). Over successive days, such a series of worker ovipositions shows an overall pattern that is made up of a phase of increasing numbers followed by a phase of decline.

Worker oviposition is likely triggered by an uptake of food, which activates the ovaries (Velthuis, 1993; see also Koedam, 1999; this study). How food can cause stingless bee queens to lay eggs without them being fertilized, like in *M. beecheii* where the queen exclusively produces the males (Paxton et al., 2001), is still not well understood. In *Melipona*, trophallaxis between the queen and the workers seems to be of minor significance for the queen's diet (Sommeijer, 1985). Instead, queens characteristically eat larval food and trophic eggs prior to oviposition (Sakagami, 1982), which means that the processes of cell provisioning allow the queens to adjust their need for nutrients to their egg-laying tempo. As a result of the favourable food conditions we induced, brood cells were produced at higher frequencies in both colonies and the workers in colony 1 produced more trophic eggs per cell. It seems therefore, that when *M. subnitida* queens lay eggs at higher frequencies, a larger proportion of these eggs will not be fertilized.

Queen-worker male production ratio

Male parentage has been determined in several stingless bee species (Contel and Kerr, 1976; Machado et al., 1984; Paxton et al., 2001; Palmer et al., 2002; Tóth et al., 2002a,b; Chinh et al., 2003; Sommeijer et al., 2003). The proportions of males produced by the queen and by the workers in *M. subnitida*, presented by Contel and Kerr, were estimates based on an isozyme analysis of males sampled from 54 colonies in the Northeast of Brazil (the natural habitat of this species). Based on behavioural observations on egg laying over a restricted period in two colonies stationed at the bee laboratory of São Paulo University and on the analysis of the resulting sex ratio, we were able to confirm that queens produced more males than all the workers together. Here, we also report that when workers start laying male eggs, the queen can share in a substantial way, and that outside this period of concentrated male production, queens are able to produce some males. Based on relatedness grounds, stingless bee workers should produce all of the males (Queller and Strassmann, 1998). We conclude, therefore, that workers in *M. subnitida* either lack complete control over male production or their male production carries a cost for the colony.

Strategies in male egg laying by workers: benefits and costs

Although a cell can be oviposited in several times, only a single individual will emerge from it (Sakagami, 1982). Based on behavioural observations and the frequency with which males emerged from cells oviposited in by the queen as well as by a worker, Beig (1972) concluded that the *S. postica* male developing from these doubly-oviposited cells is usually worker-derived (see also da Silva, 1977). As in our study, male maternity was not genetically verified. We did, however, find in 29 cases of cells with two eggs, two that gave rise to a worker, both occurring in colony 2. This shows that, in *M. subnitida*, the queen's egg is able to develop into an adult bee in some cases of doubly-oviposited cells. This means that excluding the queen at egg laying is always a more secure way for a *M. subnitida* worker to have her egg develop into a male. Cases like those reported for *Trigona subnuda* (Tóth et al., 2002a), in which cells were oviposited in by reproductive workers up to two days after they had been oviposited in by the queen and subsequently closed, were not observed in *M. subnitida*. It also never occurred that a reproductive worker replaced an egg already present in the cell with one of her own, a behaviour commonly seen in *M. bicolor* (Koedam et al., in prep.).

Koedam et al. (1999) and this study show that the *M. subnitida* queen is not able to prevent workers from laying reproductive eggs. In addition, the occasions in which workers lay an egg before the queen can oviposit in that cell are common for the species. It is exclusively in these cases that the queen taps laying workers with great vigour, a behaviour which seems to express conflict (Tóth et al., 2004). This tapping also occurs when workers close the cell following their egg laying. Especially in these cases of egg laying, workers show a strong preference to lay their next egg on another comb, generally the newest comb under construction.

An obvious side effect of the reproductive workers' behaviour to choose cells in the comb most recently under construction in which to deposit male eggs is that cells predominantly located in the centre of the combs will receive these eggs. Furthermore, the queen may accompany the reproductive workers, thus strengthening the effect of this male clustering. A positive correlation among *Melipona* species between the occurrence of male cells clustered in the centre of the combs and the occurrence of reproductive workers (Kerr, 1950; Bezerra, 1995; Koedam, 1999; Koedam et al., 1999; Paxton et al., 2001; Sommeijer et al., 2003; Quezada-Euán, pers. comm.) could mean that the tendency of *Melipona* workers to lay male eggs in the youngest comb is common.

There is general consensus about the peaceful nature of interactions between castes in Hymenoptera (Ratnieks and Reeve, 1992). In fact, in stingless bees, some agonistic behaviours, especially those occurring during the acts of cell provisioning and egg laying, are believed to have become ritualized (Zucchi, 1993). However, overt conflict, like in *P. subnuda*, does occur: the queen was seen to occasionally push a reproductive worker from the cell during egg laying (Tóth et al., 2002a).

In *Melipona*, only the workers have fighting abilities (Koedam et al., 1995); the queen's morphology, when physogastric, is adapted exclusively to egg laying. Killing or injuring the queen would prejudice the survival and reproduction of the colony (Trivers and Hare, 1976; Ratnieks and Reeve 1992; see also Hamilton, 1971). Although the workers' personal direct fitness (male production) and even immediate inclusive fitness (male production by sisters) might rise when they dispose of their queen, the loss of the queen might incur long-term losses to the colony which contributes to long-term losses to the workers' inclusive fitness. *Melipona* bees, in particular, show a high investment in colony survival and swarm infrequently (Roubik, 1989; J.C. Biesmeijer, D. Koedam and M.B. Dijkstra, unpublished work).

A struggle between two reproductive workers has been reported in *M. subnitida* (Koedam et al., 1999), while the many occasions of an apparent confrontation between the queen and a reproductive worker that prevented her from egg laying were never seen to turn into fights. There was, however, one occasion when a reproductive worker dragged the queen from the cell just when she was going to oviposit. All this shows that, although with a low incidence, workers can display overt aggression towards other reproductives, including the queen. It is not so much the frequency of this aggression, but more its intensity that poses a serious risk to the colony. We hypothesise therefore, that in *M. subnitida*, in addition to their reproductive specialisation which possibly reduces the performance of other nest duties (Bourke, 1988; Ratnieks and Reeve, 1992), the potential aggressiveness of reproductive workers is an extra cost to the colony, a cost that has not been acknowledged previously. Further research is needed to see if the case in which workers, especially those that prevent the queen from egg laying and receive her vigorous tapping, laid male eggs predominantly in the youngest combs are a consequence of the workers' efforts to maximise personal direct fitness while minimising long-term losses in inclusive fitness.

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