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Conflict over Male Production in Stingless Bees

by

Eva Tóth

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

Doctor of philosophy

APPROVED, THESIS COMMITTEE:

Joan Strassmann, Professor
Ecology and Evolutionary Biology

Dave Queller, Professor
Ecology and Evolutionary Biology

Ronald S. Sass, Professor and Chair
Ecology and Evolutionary Biology

Evan Siemann, Assistant Professor
Ecology and Evolutionary Biology

Rick Wilson, Professor
Social Sciences and Humanities

Houston, Texas
May 2002
ABSTRACT

Conflict over Male Production in Stingless Bees

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Eva Tóth

Although social hymenopteran colonies show a high level of cooperation among their members, colony members can have conflicts among themselves as well. One of these intra-colonial conflicts is who produces the males. I studied the resolution of conflict in stingless bees, a species-rich group with a tropical distribution. In the majority of stingless bee species both workers and queens are able to produce males. Therefore intracolonial conflict over male production is predicted. Because stingless bee queens mate only once, workers are more related to their own and to each other’s sons than to the sons of the queen. Thus on genetic grounds, worker production of males is expected. However, workers might not reproduce if the costs of reproduction are high, or if the queen is able to suppress workers. The decision could have been made in the bygone times and the current pattern does not serve adaptive functions at the present.
To test my predictions of conflict over male production I looked at three levels: within colonies, within species, and between species. On the colony and species level, I hypothesized that current conflict is expressed by behavioral antagonism between the workers and their queen. Furthermore, I predicted behavioral conflict to be higher in the periods when males are produced compared to periods with only female production. On the level of comparison between species I expected more signs of conflict in species where both workers and queen produce males than in species where males are all queen derived.

The conclusions of this study concerning conflict over male production in stingless bees are: 1) Genetic tools confirmed that workers reproduce in some, but not in other species. 2) The costs involved with worker reproduction could explain why in some species workers reproduce and in others not. 3) There is not only a variance of worker reproduction between, but also within species. Demographical factors might be essential determining the amount of worker reproduction within species. 4) The pattern of worker reproduction could be explained by costs although phylogenetic relationships could explain the pattern also.
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CHAPTER I

General introduction

One of the most fascinating questions in evolutionary biology is why in highly social Hymenoptera (ants, wasps and bees) most individuals do not reproduce. In many insect societies non-reproducing individuals, called workers, have partly or completely given up their reproduction to help their mother, the queen, to reproduce (Wilson 1971). As a result of this task specialization the queen is mainly the one who produces the offspring while the workers build the nests, collect and store food, raise the queen’s progeny and defend the colony against intruders often at the cost of their lives (Wilson 1971). Naturalists have long been surprised by workers that sacrifice their life for other individuals, and by how such a situation could have evolved. Darwin (1959) recognized this as a problem, seemingly disproving his theory of natural selection of which one of the cornerstones was individual reproduction. He suggested that sterile conditions could be advantageous under certain situations. Although Darwin was on the right track, he could not work out just how this could be, because he lacked knowledge of genetics.
**Hamilton’s rule**

Despite the general interest in social insects, scientists could not understand how conditions could favor sterility until Hamilton (1964) published his paper introducing the concept of ‘inclusive fitness’ and the importance of costs and benefits on one hand and relatedness on the other in the evolution of sociality. He showed that helpers’ degree of relatedness to beneficiaries could be the basis for the evolution of helping. So in his model the fitness of a sterile helper is not only defined by the number of offspring it can produce itself (i.e. direct fitness), but also by the number of offspring of the animals it helps if they are related (indirect fitness). As his simple but ingenious equation shows: $B_r b > C r_e$, where $B$ is the increase of fitness of the beneficiary, $C$ is the cost to the helper, $r_b$ the relatedness coefficient of the helper to the beneficiary and $r_e$ the relatedness coefficient of the helper toward itself. Although helping workers do not directly reproduce, they make sure their genes are preserved through the offspring of their relatives. When the costs outweigh the benefits, helping is no longer a reproductively poor strategy.

Hamilton’s rule is extensively used to explain social cohesion in insect colonies. Social Hymenoptera are a really interesting test of
Hamilton's model because of hymenopteran haplo-diploid genetic system. In this group females arise from fertilized, diploid eggs, whereas males develop from unfertilized, haploid eggs. A mated female keeps sperm in a sack-like organ, the spermatheca, and she is able to control whether she allows sperm to fertilize the egg before she lays it (Wilson 1971). A mated hymenopteran female can thus determine the sex of her offspring.

*Hamilton's rule applied*

As a consequence of the haplo-diploid system, workers in a social insect colony that is headed by one singly mated queen, like most ants, wasps, bumble bees and stingless bees, share all paternally transmitted genes and half of the maternally transmitted genes. A worker is thus on average related to her sister by $r_b = 3/4$, whereas she would be related to her daughters only by $r_c = 1/2$. Assuming that costs and benefits are the same, a worker is reproductively better off by rearing sisters than by producing daughters herself.

If the queen mates more than twice however, as in honeybees, worker relatedness to sisters declines ($r_b =$ between 1/4 and 1/8) to a relatedness value that is less, than when a worker would produce daughters herself ($r_c = 1/2$). Why workers do not reproduce under such conditions could be explained by
collective worker interests. Workers are always more related to their mother's daughters than to the daughters of their sisters. Workers might prevent each other from reproduction allowing their mother to do it (Fig 1). However, worker interest does not explain why colonies with multiply mated queens stayed together and did not fall apart when sociality evolved. Maybe multiple mating by queens evolved later in time when workers already gave up their mating capacity and could not return from this evolutionary dead end. Alternatively, harsh environmental conditions may have forced workers to stay with their mother and as a consequence of successful worker policing workers lost their mating capacity. Non-reproductive workers might indirectly reproduce more by staying with the queen than by founding their own nest. Because social insects are very successful, living together must be advantageous evolutionary strategy.

Considering male production, females give half of their genetic material to their sons ($r_c=1/2$). In honeybees where the queen mates multiply, workers are again more related to queen-produced males ($r_b 1/4$) than to worker-produced ones ($r_b$ between $1/4$ and $1/8$, Fig 2). Workers thus, collectively prevent their sisters from reproducing and allow their mother to produce the males (Woyciechowsky & Lomnicki 1987, Ratnieks 1988). In
**Relatedness of workers to:**

- **daughters**
- **sisters**
- **daughters of sisters**

**Fig 1:** Relatedness of workers to females in colonies with single queen mated to various numbers of males, each male contributing equally to brood (after Ratnieks 1988).
Fig 2: Relatedness of workers to males in colonies with a single queen mated to various numbers of males, each male contributing equally to brood (after Ratnieks 1988).
honeybees there is thus only one way the conflict can be solved. As a rule workers do not reproduce, and allow the queen to produce the males. Resolution in conflict over male productions could be different in species where the colony is headed by one, singly mated queen (Queller and Strassmann 1998, Bourke and Ratnieks 1999). Here, workers are not only more related to their own sons ($r_c = 1/2$) but also to the sons of their sisters ($r_b = 3/8$), above the sons of the queen ($r_b = 1/4$). In this case the interests of workers and their queen collide, and other factors being equal, workers should collectively help each other to produce the males acting against queen male production. Costs, however, might force workers partially or entirely to refrain from reproduction (Ratnieks & Reeve 1992). Despotic queens might control worker reproduction as well (Fletcher & Ross 1985, Foster et al. 2000).

Costs versus queen control

Although the costs in Hamilton’s equation are difficult to measure and thus express quantitatively, costs make sense and can be understood intuitively. Costs in social insects include 1) costs by damaging the queen if the conflict
escalates into a fight, 2) costs by ignoring tasks and just focusing on reproduction and 3) costs due to recognition errors.

If conflict leads to an open fight, the opponents could damage each other and one of them could die. The death of a worker does not affect the colony, since there are many of them, but the death of the queen incurs high costs (Ratnieks and Reeve 19992). When the queen dies, no females are produced before a new queen gets accepted, mates and starts to reproduce. This delay affects colony size and in extreme situations can lead to colony death. Also, a new queen being the sister instead of the mother of the workers, her progeny will be less related to the workers than the progeny of a mother queen. Queen death means thus a decrease in relatedness as well. Costs might also be severe due to ‘lazy’ workers. If most workers decide to reproduce and not to work, the colony will run out of resources and die eventually (Ratnieks and Reeve 1992). Workers can also incur costs to the colony due to recognition errors. If workers’ recognition system is not perfect and they are not able to distinguish between female and male eggs of the queen, by destroying female eggs instead of male eggs they could reduce future worker force and consequently reducing colony success (Keller 1999).
Despotic queens could also repress worker reproduction. A bigger and heavier queen might be able to physically prevent workers from reproduction by disturbing them while workers are laying their eggs, or she could destroy worker eggs after they are laid. Queen could also repress worker reproduction by pheromones. Although pheromonal suppression is not supposed to work on the evolutionary time scale, because workers can adjust their sensitivity to queen pheromones, an evolutionary arms race could evolve, in which the queen has sometimes more and sometimes less control over worker reproduction. A chemical arms race does not have to be costly to the participants (Foster et al. 2000) and thus can be a possibility. Because costs and queen control give the same results, it is difficult to tease them apart unless we have an indication for changing pheromones of the queen or unless we perform a multi-species study on worker reproduction. Depending thus on costs (different ecological factors) and/or a possible arms race the costs over male production in species with singly mated queens can result in different outcomes.

What conflicts teach us

Understanding how potential conflicts in insect colonies are resolved despite conflicting interests on the individual level helps us to understand how other
complex life forms could have evolved on earth (cells with different organelles, multi-cellular organisms and societies, Maynard Smith & Szathmáry 1995). Species with a once mated queen are thus of particular interest because the conflict over male production can be resolved in different ways depending on the costs and benefits in different situations.

_The appropriateness of stingless bees for studies of conflict_

Stingless bees are excellent study organisms to study colliding interest between the workers and their queen because most species have one, singly mated queen (except for *Melipona bicolor* which often has more than one queen), creating a conflict of interest between the workers and the queen over who should produce the males (Peters et al. 1999). The group is species rich, with more than 400 known species (Michener 1974) which makes comparative studies possible. Stingless bees reached a high degree of sociality where workers gave up the production of females completely and can not exist independently of the colony (Michener 1974). Whether a real conflict between workers and queens occurs can not only be tested with genetic methods but can also be confirmed by analyzing behavioral interactions between the workers and their queen. Whereas genetic outcome can only suggest that there a conflict, it can not give an answer for whether
the conflict is settled or not. Behavioral observations are needed to confirm an ongoing conflict. Stingless bees have an elaborate oviposition process requiring the presence of both workers and the queen facilitating behavioral observations (Zucchi 1993). Furthermore, stingless bees have mass provisioning of cells; a cell is filled with larval food first before an egg is laid in it, after which it is closed (Engels & Imperatriz Fonseca 1990). This makes worker or queen policing of cells once they are closed difficult. Although queens usually consume worker eggs, their mandibles are generally not strong enough to open a cell after it is properly closed (Schwarz 1948, Sakagami 1982). Workers on the other hand, usually do not consume eggs in queenright (when the have a functional queen) conditions (Zucchi 1993). When workers reproduce they might lay another egg next to the one, already laid on the larval food, but do not remove or eat the first egg (except for *M. bicolor*). All the above mentioned properties make stingless bees superb for studying social conflicts and how those conflicts are resolved.

*General biology of stingless bees*

Stingless bees are confined to tropical areas of four continents (America, Asia, Africa, Australia), with the Neotropics as the most species rich area
(Sakagami 1982). They have diverse nesting sites with species nesting underground, in tree trunks, on branches and in termite nests. Stingless bees can build their cells arranged in an amorphous cluster or, as most species do, arrange the cells in a regular way (Fig 3) forming horizontal combs where a newer comb is built on top of the older ones (Sakagami 1982). Cells can be built and oviposited one by one (asynchronous) or in batches (synchronous, Fig 4). Individual cells can be provisioned, and laid temporally separated from each other (provisioning and oviposition process, POP), provisioned and laid in groups (integrated oviposition process IOP) or facultatively oviposited (if one cell is ready one cell is oviposited if several cells are ready they are all oviposited in a short time)(Zucchi 1993). All the cells/combs are concentrated in one region of the nest, which is called the brood area. Around the brood are the egg-shaped storage pots that are either filled with pollen or honey (Sakagami 1982).

For the study of how genetic conflicts are resolved the brood area is the most important place. New cells are built on the edge of the existing combs by a group of workers. The queen usually visits the new comb several times during the building process. In species with POP when the cell is ready several workers and the queen collect around it (Fig 5) and interact with each other in a species-specific way. This worker-queen interaction is
Fig 3: Brood cells built in a cluster and in a regular way
Fig 4: Asynchronous and synchronous way of comb building
often described in terms of conflict (Zucchi 1993). The workers often dart at
the queen, inspect and dive into the cell, the queen in turn inspects the cell
several times and tries to tap workers’ back with her fore legs regularly.
With time, movements become more frequent until a worker dives into the
cell and regurgitates larval food in it. Other workers follow the first worker
until the cell is almost full. Then one or several workers might lay a trophic
egg on the fluid, which is consumed by the queen. The queen subsequently
lays her egg on the larval food, and leaves the cell. The cell is closed by a
worker that usually puts her abdomen in the cell and performs rotational
movements, pushing the cell wall inwards while using her abdomen as solid
surface to push against (Zucchi 1993). Species that have IOP build many
cells at the same time and when the cells are ready the queen patrols over the
comb’s edge (where the new cells are) inspecting the cells. After a while a
general arousal spreads over the comb and cells are synchronously
 provisioned by workers. The queen then goes from cell to cell eating trophic
eggs if the cells contain them, laying her own eggs on the larval fluid
afterward (Zucchi 1993). Workers subsequently close the cells and are often
disturbed by the queen, who still patrols over the comb for a few minutes,
searching for other cells available for laying.
Fig 5: The queen and workers around an empty cell (after Sakagami 1982)
Worker eggs and reproductive strategies

Workers in stingless bees can lay two types of egg, trophic and reproductive eggs (Sakagami et al. 1963). Trophic eggs are always laid before queen oviposition and are consumed by the queen. These eggs do not have a nucleus and are not viable (Koedam 1994). It is very likely that trophic eggs are meant to feed the queen, however, trophic eggs could also be worker eggs, laid prematurely because of queen manipulation. Reproductive worker eggs can be laid in four different ways depending on the species. A worker can reproduce before queen oviposition, after which the worker closes the cell before the queen can get to it (Sommeijer et al. 1984b). A worker can lay a reproductive egg also right after queen oviposition after which the worker leaves the cell and another worker closes it (Beig 1972). Workers can lay eggs in a cryptic way while closing the cell and their abdomens are in it (Beig 1972). Workers can reopen already laid and closed cells to lay an egg in it (Koedam et al. 1999). The queen in turn can push workers away from the cell and eat their eggs, but is unable to open properly closed cells (Sommeijer et al. 1984).
The purpose of the study

As argued above, a potential conflict exists between the workers and the queen in stingless bee colonies because the colonies have just one singly mated queen. In this study I investigated whether this potential conflict becomes a real conflict and manifests itself in 1) worker reproduction and 2) behavioral conflicts between the workers and the queen. The outcome of conflict over male production was analyzed on three levels: within colonies, within species and between species. On the colony and same species level we expected to see more behavioral conflict occurring between the workers and the queen during the POP in the time when males are produced compared to periods when no males are produced. When comparing different species with each other we expected more behavioral conflicts occurring in species where both workers and queens produce males than in species where only one of the castes produces the males. Comparing different species on basis of genetic analysis of male production gives us an opportunity to speculate why the patterns of male production differ between species. Different species differ in their oviposition processes, colony sizes and the size of the queen versus the workers. These all could have a role in the outcome of who is producing the males.
Outline of this Thesis

In Chapter 2 I specify methods that were not described in detail in the following chapters, giving our recipe of DNA extraction, and PCR reactions.

In Chapter 3 I investigate the occurrence of male production by workers in eight stingless bee species. I also compare different methods of assessing the amount of male production by the queen and the workers.

Chapter 4 deals with the predictions that apply for male production and the level of aggressivity within colonies and between colonies of the same species. In this chapter I compare behavioral worker-queen interactions in Schwarziana quadripunctata quadripunctata before and during the male production period.

In Chapter 5 I also investigate on the colony and same-species level, but work with Paratrigona subnuda, a species where workers contribute to male production. I address the question whether the queen is able to control worker reproduction and address the problem why workers do not completely dominate male production.

In Chapter 6 I compare different species' degree of 'aggressivity' during the oviposition process and investigate whether it correlates with the degree of conflict between the workers and the queens over male production.
Chapter 7 is also a comparative study between species. It deals with how male production is determined in stingless bees and what different forces could influence the results of conflict over male production.

In Chapter 8 I synthesize the results obtained from the four previous chapters.
CHAPTER II

General methods

_Housing, videotaping and collection of bees_

Observation hives were inside a building and consisted of a heated wooden outer temperature-controlled box (28°C) and a smaller inner box that contained the colonies (Sakagami 1966). The colonies were connected to the outside by a plastic tube, so that the bees could forage and normal colony activity would not be disturbed (Fig 1). Both the colony-box, and the thermo-box were covered by glass plates on the top, making videotaping of colonies possible. We used red or cold lights (not heating the colony) to illuminate the colonies to avoid disturbance during videotaping. When the nests were not being observed we covered the thermo box with a black cloth and Styrofoam plates to prevent disturbance of bees by light and to prevent the cooling off of colonies.

The cover glass plates had one or two windows, and those windows were themselves covered with smaller glass plates (Fig 1). When we collected bees we darkened the room where the colonies were, and used red
Fig 1: The housing of the observed colonies
lamps (bees do not see red) to illuminate the nests. We removed the small glass plate without causing a big disturbance and collected individual bees through this window using a suction tube. From colonies that were not in observation hives, we removed the lid of the hive and collected bees as quickly as possible and closed the hive again. The collected bees were then chilled at $-7^\circ$C for a couple of minutes. After the bees were immobilized we sorted out the young males and females we needed, and reintroduced the rest of them into their colony. Chilling did not affect the bees over the long term, after approximately half a minute the introduced bees started to move and after about a minute they could walk again. The collected individuals were put in 100% ethanol while still chilled.

**DNA extraction of individuals**

We made 50ml grinding solution that contained 0.1M NaCl, 0.1M Tris-HCL (pH 9.1), 0.05M EDTA, 0.05% SDS. We added 500µL grinding buffer and bee thoraxes in individually numbered 1.5 mL eppendorf tubes and ground the toraxes fine with minipestels (Strassmann et al. 1996). We incubated the homogenized tissues in grinding buffer for 30 minutes in a 65°C water bath. After that we added 86µL of 8M potassium acetate to the tubes, mixed their
content and put them at 4°C for 30 minutes. We then spun the tubes at 10,000 rpm for 5 minutes.

We transferred as much as possible supernatant to a new set of labelled 1.5 mL tubes. Added 2.5 volumes of cold 100% ethanol into the tubes, mixed their content and incubated them at -20°C overnight. The next day, we spun the tubes at 10,000 rpm for 5 minutes and discarded the supernatant. We dried the DNA and resuspended it in 500-1000 μL of sterile ultrapure water according its quality and quantity, that we assessed visually after running a part of the DNA out on an agarous gel and staining it with ethidium bromide. At the end we stored our DNA at 4°C.

**PCR reactions**

We used PCR reactions to amplify fragments of interest in the extracted DNA. The mix for PCR is given in Table 1. The used primers were originally designed for *Melipona bicolor* (Peters et al. 1998) and *Scaptotrigona postica* (Paxton et al. 1999). We programmed the PCR machine for DNA fragment amplification at the optimal annealing temperature for each primer (Table 2) for 40 cycles after which we stored our samples at 4°C.
Separation and visualization of DNA fragments

We added 5 μL stop solution (Strassmann et al. 1996) to our PCR samples and denatured them at 95°C for 5 minutes. We electrophoresed the PCR samples on 6% denaturing polyacrylamide gels at 100Watts. We visualized PCR products by exposing the dried gel to an X-ray film. The length of our trinucleotide fragments was assessed using M13 sequencing reactions as size standards. The products were double scored to reduce scoring errors. Samples with disagreement or that did not amplify were rePCRed and rerun.
Table 1: Recipe of the grinding buffer for DNA extraction (according to Strassmann et al. 1996)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock</th>
<th>1μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH2O</td>
<td>------</td>
<td>3.02</td>
</tr>
<tr>
<td>10x buffer</td>
<td>------</td>
<td>1.00</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>25 mM</td>
<td>0.62</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>10 mM</td>
<td>0.10</td>
</tr>
<tr>
<td>Primer</td>
<td>2.5 mM</td>
<td>2.00</td>
</tr>
<tr>
<td>genomic DNA</td>
<td></td>
<td>3.00</td>
</tr>
<tr>
<td>Taq Pol</td>
<td>5 U/μl</td>
<td>0.10</td>
</tr>
<tr>
<td>35S dATP</td>
<td>12.5 μCi/μl</td>
<td>0.16</td>
</tr>
<tr>
<td>TOTAL</td>
<td>------</td>
<td>10.00</td>
</tr>
</tbody>
</table>

Table 2: Programming of the termo-cycler for multiplying DNA fragments extraction (according to Strassmann et al. 1996)

<table>
<thead>
<tr>
<th># Cycles</th>
<th>Temp. 1</th>
<th>Time 1</th>
<th>Temp. 2</th>
<th>Time 2</th>
<th>Temp. 3</th>
<th>Time 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>92°C</td>
<td>30 sec.</td>
<td>Tₐ</td>
<td>30 sec.</td>
<td>72°C</td>
<td>45 sec.</td>
</tr>
</tbody>
</table>
CHAPTER III

Male production in stingless bees: variable outcomes of queen- worker conflict
Introduction

Social insect colonies are characterized by a high degree of cooperation among their members (Wilson 1971, Michener 1974). Cooperation could have evolved because colony members are often related and share their genes (Hamilton 1964). Because individuals are not genetically identical, however, insect colonies have to cope with conflict situations as well (Trivers & Hare 1976, Ratnieks & Reeve 1992, Queller & Strassmann 1998, Bourke & Ratnieks 1999). Kin selection theory not only explains cooperation among relatives but also explains how selfish behavior leading to conflicts can take place (Hamilton 1964, 1971).

Conflicts in insect societies often arise because females are not uniformly related to male offspring. Hymenoptera have a peculiar sex differentiation system where females hatch from diploid, fertilized eggs while males are produced by haploid, unfertilized ones. While all females are equally related to their own sons (r=1/2), they are less related to other females' sons. In insects with a single queen that mated many times, such as honeybees, workers are on average less related to other workers' sons (r between 1/4 and 1/8) than to sons of the queen (r=1/4). As a result honeybee workers police each other and prevent each other from reproducing, whereas
they collectively allow their mother, the queen, to produce the males
(Woyciechowsky & Lomnicki 1987, Ratnieks 1988, Ratnieks and Visscher
1989). In contrast, in species where the queen mates just once, such as
stingless bees (Silva et al. 1972, Contell & Kerr 1976, Peters et al. 1999)
workers are more related to other workers’ sons (r=3/8) than they are to the
sons of the queen (r=1/4). Therefore, on relatedness grounds workers should
allow each other to reproduce and collectively oppose queen production of
males (Queller and Strassmann 1998).

Previous behavioral research has indicated that there is some variation
in whether the workers or the queen produce male eggs in stingless bees
(Beig 1972, Sommeijer et al. 1984a, van Benthem et al. 1995, Inoue et al.
1999, Koedam et al 1999, Grosso et al. 2000) even though the single, once-
mated queen rule appears to be general across the tribe (Peters et al. 1999).
Multiple mating reported for three species (Machado et al. 1984, Paxton et
al. 1999) has either not been supported by further work or was limited
even to keep effective mate numbers close to one (Paxton et al. 2001).

There is no worker reproduction in a few species whose workers
never have developed ovaries (Suka & Inoue 1993, Sakagami et al. 1963,
Sakagami & Zucchi 1974). However, some behavioral observations suggest
that, even in species where workers have developed ovaries, they might not
always lay male eggs (Sommeijer et al. 1984b, Inoue et al. 1999, Grosso et al. 2000, Suka and Inoue 1993, Sakagami & Zucchi 1974). However, behavioral data are imperfect for two reasons. First, workers sometimes lay their reproductive eggs quickly and secretly, while covering the cell with their abdomen, outside the normal oviposition process (Chapter 5). Second, workers who are observed laying eggs may not actually reproduce because workers often produce trophic eggs lacking nuclei to feed the queen (Sakagami 1982). So observation of worker egg production is often not sufficient to infer worker reproduction.

Clearly this is an area where genetic markers could be useful. Some genetic studies have augmented the behavioral data (Contel & Kerr 1976, Machado et al. 1984, Drumond et al. 2000, Paxton et al. 2001, Chapter 4, 5). But even here the results can be ambiguous. Males who are the progeny of either the workers or the queen can only be excluded for the latter, because queens have a subset of worker genes. If non-excluded males are attributed to the queen, exclusion studies therefore risk underestimating worker production.

Here we report male production data for eight species of stingless bees employing DNA microsatellite markers and a likelihood procedure that is unbiased with respect to worker versus queen production. The primary
goal of our study was to investigate parentage of male brood in a number of species to get a more general test of the prediction that workers will contribute to male production. A secondary goal is to see to what extent the likelihood procedure improves on simple exclusion methods.

Materials and Methods

Species collection

We collected worker and male samples from the following species: *Melipona marginata, M. quadrifasciata, M. scutellaris, Tetragona clavipes, Scaptotrigona postica, Plebeia droryana, P. remota* and *P. saiqui*. All the samples were collected in Brazil between March and May 1999 and between October and November 1999 in São Paulo, Brazilia, and Bahia provinces. The colonies were respectively the property of The University of São Paulo, Dr. Paulo Nogueira Neto, and the University of Salvador. All colonies were kept in wooden hives. We collected specimens by opening the hives and gathering young adults with a suction tube. If the upper comb in the colony was ready to hatch we removed a part of it to collect pupae as well. The
collected bees were chilled and then transferred into tubes with 100% ethanol and kept at 4°C until further analysis.

Genetic data

We genotyped workers and males in a various number of colonies per species with a total of 454 workers (9 to 20 per colony) and 412 males (from 5 to 29 per colony). We genotyped a total of 17 moderately polymorphic microsatellite loci (Peters et al. 1998, Paxton et al. 1999), that had two to eight alleles per species (Table 1). We extracted DNA, set up polymerase chain reactions (PCR) and visualized PCR products on polyacrylamide gels following the protocols of Strassmann et al. (1996). To minimize error, gels were independently scored twice (by two people). The results were compared and discrepancies were reconciled, a process that sometimes included redoing the genotype entirely. To estimate relatedness within colonies for our microsatellite data we used the computer program Relatedness 5.07 for Macintosh (Queller & Goodnight 1989; Goodnight and Queller 2000). Standard errors were based on jackknifing over loci for within-colony estimates and over colonies for population estimates (Queller & Goodnight 1989). We weighted colonies equally in all analyses. We
calculated 95% confidence intervals (C.I.) assuming that the jackknifed pseudo-values followed a t-distribution.

To estimate the proportion of males that were produced by the queen or the workers we used a likelihood method (Chapter 4) which is a modified version of that used by Arévalo et al. (1998), and was designed for multiple-queen species. For singly mated species, we have a set of worker and male genotypes, and we infer queen genotypes from the workers. Let $Q$ be the fraction of males that come from the queens. We test various hypothetical values of $Q$ to see which has the highest likelihood of having produced the observed male data (in practice, we test all values between 0 and 1 at increments of 0.02). The likelihood, $L$, for any value hypothetical value of the $Q$ is calculated as $L = K \prod_{\text{males}} \left( Q \prod_{\text{loci}} f_{qi} + (1 - Q) \prod_{\text{loci}} f_{wi} \right)$. $K$ is a multinomial constant that never has to be calculated because it multiplies all $L$'s by the same value, and cancels out of any comparisons. For each male allele considered in turn, $f_{qi}$ and $f_{wi}$ are the frequencies of that allele in the queen and in the workers.

One complication arises when workers are all heterozygous, because then we do not know which allele came from the queen and which from her mate. In that case, we average the likelihoods of both possibilities, weighted by their likelihoods of occurring (if the population frequencies of A and B
are $p$ and $q$ respectively, an AA x B mating has likelihood $p^2q$ and a BB x A mating has likelihood $pq^2$, so their relative likelihoods are $p$ and $q$.

Calculating the two likelihoods separately in this way, and then averaging, is the best method because one of the two matings is correct and it applies to all offspring; it makes no sense to use one mating for some offspring and the other mating for the rest. An excel spreadsheet to apply these calculations is available from DCQ.
Table 1: The primers used for genetic analyses for the species included in the study and their polymorphism. The number of colonies included in the relatedness estimations and the number of workers included in the study, the number of colonies that were sampled for males and the number of males that was genotyped per species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Primers used (alleles)</th>
<th>Total colonies</th>
<th>Number of workers</th>
<th>Colonies with males</th>
<th>Number of males</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Plebeia saiqui</em></td>
<td>Mbi33 (3), Mbi215 (3), Mbi254 (3), Mbi259 (3), Mbi278 (7), G38805 (7)</td>
<td>4</td>
<td>36</td>
<td>3</td>
<td>66</td>
</tr>
<tr>
<td><em>P. remota</em></td>
<td>Mbi215 (2), Mbi259 (4), G38805 (3)</td>
<td>7</td>
<td>79</td>
<td>5</td>
<td>83</td>
</tr>
<tr>
<td><em>P. doryana</em></td>
<td>Mbi11 (2), Mbi522 (2)</td>
<td>2</td>
<td>22</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td><em>Scaptotrigona postica</em></td>
<td>Mbi254 (6), Mbi259 (5), Mbi278 (3), G38803 (6), G38804 (7), G38805 (7), G38807 (4)</td>
<td>5</td>
<td>44</td>
<td>3</td>
<td>62</td>
</tr>
<tr>
<td><em>Melipona quadricapitata</em></td>
<td>Mbi201 (6), Mbi232 (7), Mbi254 (6), Mbi259 (4)</td>
<td>3</td>
<td>39</td>
<td>2</td>
<td>47</td>
</tr>
<tr>
<td><em>M. scutellaris</em></td>
<td>Mbi28 (3), Mbi213 (3), Mbi232 (4), Mbi254 (7), Mbi256 (2), G38805 (3)</td>
<td>9</td>
<td>89</td>
<td>5</td>
<td>46</td>
</tr>
<tr>
<td><em>M. marginata</em></td>
<td>Mbi28 (3), Mbi32 (3), Mbi232 (5), Mbi201 (4), Mbi259 (3), Mbi278 (6)</td>
<td>5</td>
<td>51</td>
<td>3</td>
<td>41</td>
</tr>
<tr>
<td><em>Tetragonula clavipes</em></td>
<td>Mbi232 (8), Mbi259 (3), Mbi278 (2), G38805 (2)</td>
<td>10</td>
<td>94</td>
<td>5</td>
<td>47</td>
</tr>
</tbody>
</table>
Results

Within colony relatedness

As expected, workers in all species and colonies appeared to be full sisters (Fig 1). We did not observe more than three alleles per locus per colony. All workers always shared one allele at every locus from the queen's haploid mate, and they possessed at most two additional alleles from the diploid queen. These data thus extend the observation that stingless bee queens mate only once (Silva 1972, Peters et al. 1999, Strassmann 2001). All 95% confidence intervals of genetic relatedness within colonies included the pedigree value of 0.75 except for *P. droryana*, which had only a single colony, and two biallelic loci that both gave point estimates of \( r = 1.0 \) (Table 2). *T. clavipes* had a comparatively low point estimate owing to two colonies with low relatedness estimates because the alleles for those colonies happened to be very frequent in the population.

Workers produced some of the males in six of the eight species (Fig 2). In a few colonies, workers apparently produced all of the males (maximum likelihood \( Q = 0 \)), but in no species was this true overall. Variation among colonies was common. Both *Melipona scutellaris* and *Tetragona clavipes* appeared to include at least one colony with complete worker production, at
Fig 1: Relatedness values (±95% CI) between workers of eight stingless bee 

*Male production*
Fig 2: Curves of relative likelihoods for the fraction of queen-produced males for colonies of eight stingless bee species. Each curve represents one colony, except for the two heavy-line curves of *P. remot*a, each of which represents two curves.
Table 2: Male production by queens as estimated by exclusion of queens and by likelihood. Colonies where both the likelihood and the exclusion method agreed that $Q = 1$ are excluded.

<table>
<thead>
<tr>
<th>Species</th>
<th>Colony</th>
<th>Males excluded</th>
<th>Males genotyped</th>
<th>Exclusion $Q$ estimate</th>
<th>Likelihood $Q$ estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. remota</em></td>
<td>colony 3</td>
<td>1</td>
<td>10</td>
<td>0.90</td>
<td>0.80</td>
</tr>
<tr>
<td><em>S. postica</em></td>
<td>colony 1</td>
<td>1</td>
<td>15</td>
<td>0.93</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>colony 2</td>
<td>7</td>
<td>18</td>
<td>0.61</td>
<td>0.58</td>
</tr>
<tr>
<td><em>M. quadriplanata</em></td>
<td>colony 1</td>
<td>0</td>
<td>22</td>
<td>1.00</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>colony 4</td>
<td>0</td>
<td>24</td>
<td>1.00</td>
<td>0.32</td>
</tr>
<tr>
<td><em>M. scutellaris</em></td>
<td>Colony 1</td>
<td>2</td>
<td>6</td>
<td>0.67</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>colony 5</td>
<td>11</td>
<td>13</td>
<td>0.15</td>
<td>0.00</td>
</tr>
<tr>
<td><em>M. marginata</em></td>
<td>colony 1</td>
<td>2</td>
<td>11</td>
<td>0.82</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>colony 2</td>
<td>4</td>
<td>16</td>
<td>0.75</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>colony 3</td>
<td>8</td>
<td>18</td>
<td>0.56</td>
<td>0.40</td>
</tr>
<tr>
<td><em>T. clavipes</em></td>
<td>colony 1</td>
<td>0</td>
<td>10</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>colony 2</td>
<td>0</td>
<td>9</td>
<td>1.00</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>colony 3</td>
<td>0</td>
<td>7</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>colony 5</td>
<td>0</td>
<td>11</td>
<td>1.00</td>
<td>0.44</td>
</tr>
</tbody>
</table>
least one with complete queen production, and at least one mixed colony.

These were two of the three species with the largest number of colonies sampled (5) so it is possible that some other species would have shown similarly diverse patterns with more complete sampling.

The steepness of the likelihood curves gives an indication of the range of plausible values. For example, some of the *Tetragona clavipes* curves are rather shallow. The maximum likelihood of $Q = 0.44$ in one colony is no more than twice as likely as either of the two extremes $Q = 0$ or $Q = 1$. At the other extreme, the all-queen production colonies of the three *Plebeia* species have quite steep curves, indicating that values of $Q$ much less than 1 are quite implausible.

There was some consistency among the genera represented by more than one species. There was no worker production of males in *Plebeia saiqui* and *P. droryana*, and only a small amount in one of the colonies of *P. remota*. In *Melipona scutellaris*, *M. quadrifasciata* and *M. marginata*, workers contributed significantly to male production.

Attributing males to queen when they are not only excluded as queen progeny would have missed a significant proportion of the worker production by males. Table 2 shows the maximum likelihood estimates along with the parallel exclusion estimates. Colonies for which both methods
attributed all males to queens \( Q = 1 \) are omitted. Presumably, with increasingly good genetic information, all non-excluded males would in fact be queen progeny and the two methods would give very similar point estimates. With the level of information available in this study, the likelihood method sometimes detects considerably more worker reproduction. The most extreme case is colony 3 of *Tetragona clavipes*, where the exclusion method detected no worker offspring even though the best estimate is that all males were worker-produced. In this case, there were no loci informative for exclusion because two were AA x A and the other two were AB x A. However, at the latter loci most of the males had the A allele, closely matching the 3/4 expectation under worker reproduction.

**Discussion**

Worker policing theory predicts that, if workers control who reproduces and relatedness is the crucial factor, workers should produce males in singly mated species and queens should produce them in multiply mated species. In multiply mated honeybees, it is well documented that queens produce the males, with the aid of worker policing of any worker
laying (Ratnieks 1988, Ratnieks & Visscher 1989). The other highly
eusocial bees, the stingless bees, are singly mated and therefore provide an
interesting comparison. Much prior evidence suggests that workers produce
males in this group (Beig 1972, Sommeijer et al. 1984b, Imperatriz Fonseca
& Matos 1996, Inoue et al. 1999, Koedam et al. 1999), but there have been
rather few genetic studies (Contel & Kerr 1976; Machado et al. 1984;
Drumond et al. 2000, Paxton et al. 2001, Chapter 4, 5) to confirm this.
These genetic studies suggest that workers sometimes produce males, but
not always.

Previous genetic studies have used a variety of methods to infer who
produces the workers. Paxton et al. (2001) apparently used exclusion of
queen maternity, coupled with some calculations of the probability of
missing worker maternity if it really existed. Our data show that exclusion
may miss considerable worker reproduction, though presumably this
problem is reduced as the number of loci and alleles increases. Drummond et
al. (2000) calculated expected distributions of male genotypes under queen
and worker laying and compared these with observed distributions. This
procedure is not really designed to obtain a point estimate, but does help
choose between mostly queen or mostly male production. The approach we
use in this paper is closer in spirit to that used in earlier Brazilian studies.
(Contel and Kerr 1976; Machado et al. 1984). Using a single locus, they obtained a point estimate of the queen fraction in each colony, based on the number of clearly worker-produced males, and scaled up for the fraction of worker-males expected to be missed. However, advantages of our method include incorporation of data from multiple loci, effective use of ambiguous matings (AA x B versus BB x A), and some measure of confidence in the point estimate. Good estimates are those where the likelihood curve falls off steeply. For example, the assertion that some workers and queen share reproduction within colonies of *Tetragona clavipes* is not very solid, while the same assertion can be made with confidence for *Melipona marginata*.

Our results show that workers produce some of the males in six of the eight species studied. So there is clearly an average difference, in the predicted direction, from honeybees' complete queen production, suggesting that policing theory may be on the right track. On the other hand, the prediction that workers should always produce the males is clearly not upheld. In two species, queens appeared to produce the males, and in the other six species queens produced some of the males.

The partial failure of the simple relatedness prediction does not necessarily mean that the basic policing theory is incorrect, except in its simplest form. It does imply either that workers are not in complete control
or that workers must also weigh costs and benefits rather than just relatedness. Worker laying may entail some costs to the colony, for example, a reduction in foraging or other work (Bourke 1988, Ratnieks & Reeve 1992). Workers may be unable to distinguish the queen's male eggs from her female eggs destined to be workers (Nonacs & Carlin 1990), in which case worker male production would come at the cost of reduced worker number (Ratnieks & Reeve 1992). This cost would be somewhat reduced in *Melipona* because many of the female eggs develop into queens, and most of these are not needed (Engels & Imperatriz Fonseca 1990, Ratnieks 2001). This might explain why worker production of males is common in *Melipona*, including the three species we studied, as well as *M. subnitida* (Contel & Kerr 1976), though *M. beecheii* is an exception (Paxton et al. 2001).

Not only is there variation of worker reproduction between species, but also within species. Four of the five genetic studies besides this one found similar levels of variation, for *M. subnitida*, *P. droryana*, *Scaptotrigona postica* and for *Paratrigona subnuda* (Machado et al. 1984, Contel and Kerr 1976, Paxton et al. 2001, Chapter 5). The variation within species could be explained by the costs connected to worker reproduction in some species but it is also consistent with the hypothesis that there is a
continuing evolution battle where neither participant always wins. Future studies need to decide which one of these hypothesis apply to stingless bees.
CHAPTER IV

Queens, not workers produce the males in the stingless bee *Schwarziana quadripunctata quadripunctata*
Introduction

Highly social Hymenoptera live in colonies with most females serving as non-reproducing helpers (Wilson 1971, Michener 1974). Genetic relatedness helps explain the evolution of cooperation in social insects (Hamilton 1964, 1972), but unequal relatedness between colony members can also be a source of conflict (Trivers & Hare 1976, Queller & Strassmann 1998, Bourke & Ratnieks 1999).

In Hymenoptera, where males arise from unfertilized, haploid eggs, and fertilized, diploid eggs produce females, an important issue of conflict is who produces the males. Queens and workers are most related to their own sons and less related to each other's sons. Who gets to produce the males in a colony may be resolved in favor of collective worker interests (Starr 1984, Woyciechowsky & Lomnicki 1987, Ratnieks 1988). Where the mother queen mates more than twice, as in the honeybee, workers will be more related to their queen's sons (r=1/4) than to their sisters' sons (average r near 1/8). Thus in honeybees, collective worker interests will favor queen's sons above worker sons and workers should police each other (Woyciechowsky & Lomnicki 1987, Ratnieks 1988, Oldroyd et al. 2001). Indeed in queenright
situations (when the colony has a functional queen) most honeybee workers
do not reproduce and have non-functional ovaries (Ratnieks & Visscher
1989, Ratnieks 1988). Workers with developed ovaries are attacked
(Sakagami 1954, Visscher & Dukas 1995) and most of the eggs they lay are
removed from the cells by other workers (Ratnieks and Visscher 1989). In
contrast, in species were the queen typically mates only once, as in stingless
bees (Camargo 1972, Contel and Kerr 1976, Machado et al. 1984, Olivera
Campos 1990, Peters et al. 1999), the workers are more related to their own
sons (r=1/2) and to sister-produced nephews (r=3/8) than to queen-produced
brothers (r=1/4). Based on relatedness alone, workers should thus
collectively oppose queen production of males and allow each other to
reproduce (Ratnieks 1988, Bourke & Franks 1995, Peters et al. 1999). A
potential conflict (Ratnieks & Reeve 1992) between the workers and the
queen over male production is thus expected in stingless bees.

Differences in genetic make-up in honeybee and stingless bee colonies
could account for many differences between the two groups (Peters et al.
1999). Theory predicts that stingless bee workers will be more likely to lay
eggs than honeybee workers and will therefore be in conflict with the queen.
In most stingless bees, workers have ovarian development and retain the
ability to produce haploid eggs (Sakagami et al. 1963). A fraction of those
eggs may be trophic, inviable eggs that are usually laid before queen oviposition and probably are meant to feed the queen (Crespi 1992). However, some of worker eggs are viable and can develop into males.

The best way to find out whether genetic conflict becomes a real conflict and thus manifests itself in behavior is to study the provisioning and oviposition process (POP) in stingless bees. A POP is a complex and ritualized set of behaviors between workers and the queen. It begins with an empty cell and results in a provisioned cell filled with larval food provided by the workers, and an egg which is in most cases produced by the queen (Sakagami 1982, Engels & Imperatriz-Fonseca 1990, Zucchi 1993). Many behaviors during the POP seem to be agonistic between the workers and the queens and are often described in terms of conflict (Engels & Imperatriz-Fonseca 1990). For example, in Scaptotrigona postica, where both the workers and the queen produce males, workers dart and lunge at the queen in a very aggressive way and often barricade the cell with their bodies so that the queen cannot get to it (Sakagami & Zucchi 1963, da Silva et al. 2001). However, apparently aggressive behaviors might have some other function. One way to test their agonistic nature is to see if they are more intense at times of higher predicted conflict (e.g. during male production).
In the present paper, in order to predict collective worker interests on male production, we confirmed that the queen in *Schwarziana quadripunctata quadripunctata* is singly mated, as found by Peters et al. (1999). We predicted that under single mating, all else being equal, workers would try to monopolize, or at least contribute to, male production. We tested the accuracy of this prediction using microsatellite genotyping. Finally, we tested the prediction that, if behavior during POP represents queen-worker conflict, it should be more intense during periods of male production.

**Methods**

*Study animals and data collection*

*Schwarziana quadripunctata quadripunctata* (Lepeletier) nests exclusively underground, at a depth of 0.3-3.0 m below the surface (Schwarz 1948, Imperatriz-Fonseca 1973, Camargo 1974). The bees enter their nest through a narrow, winding tube leading to the main nesting chamber. This chamber contains a brood pile of 5-8 horizontal combs, with the youngest combs on top, surrounded by 20-50 storage pots. A colony consists of one functional
queen, up to 800-2500 workers, and sometimes males and a few virgin queens. The nesting chamber is surrounded by several protective sheaths giving the nest the appearance of a ball. This makes nest collection relatively easy. A nest can be harvested in one piece avoiding mechanical damage of brood piles and pots or the loss of many bees. We dug up the nests near Cotia, Brazil (23°39'S, 46°56'W), a settlement in the Atlantic Rainforest. The nests were transported to the bee laboratory at the University of São Paulo, São Paulo City, Brazil and transferred into wooden observation hives. These hives were inside the building and consisted of a heated wooden outer thermo box (28°C) and a smaller inner box that contained the colonies. Both boxes were covered by glass on the top. For further details see Sakagami (1966). When the nests were not being observed we covered the thermo box with a black cloth and Styrofoam plates. The colonies were connected to the outside by a plastic tube, so that the bees could forage normally.

For the genetic analysis we sampled workers from 18 different S. quadripunctata quadripunctata nests. Sixteen of these nests produced males so we collected males from those nests, from February 1997 to May 1999. The collected bees were chilled and then transferred into tubes with 100% ethanol and kept at 4°C until further analysis.
We videotaped POPs from six nests between March and May 1999. We used red or cold lights (to avoid heating colonies) to illuminate the colonies and minimize further disturbance during videotaping. We videotaped POP in four colonies when they were not producing males. After this, since males are normally produced when colonies have plenty of food supplies (Bego 1990, Grosso et al. 2000, Moo-Vale et al. 2001) we attempted to induce male production by regularly supplying the colonies with extra pollen for eight weeks. Two of these four colonies did produce males (first observed 39 days and 42 days after pollen supplementation started). We also added two more colonies, which were in their male production phase from the field. Thus we had behavioral data of two colonies in their male production phase, two colonies outside of male production and two colonies in both phases.

**Genetic data, relatedness and male parentage estimation**

We genotyped samples of workers and males at seven moderately polymorphic microsatellite loci: Mbi201AAG, Mbi215AAT, Mbi219AAT, Mbi232AAG, Mbi254AAG, Mbi259AAG and Mbi278AAG (Peters et al. 1998). In *S. quadripunctata quadripunctata* these loci had three to seven alleles. We genotyped 7-15 workers and 11-39 males per colony, for a total
of 189 workers and 314 males. Whenever it was possible (but not always) we tried to collect young bees since young bees were very unlikely to have been drifters from other colonies. We followed protocols of Strassmann et al. (1996) for DNA extraction, polymerase chain reactions and visualization of PCR products of the polyacrylamide gels on autorads. Two people independently scored the autorads and their scores were compared. Discrepancies were rechecked and if necessary the sample was rerun. We used the computer program Relatedness 5.07 for Macintosh (Goodnight & Queller 2000, available at http://Gsoft.smu.edu/GSoft.html) for relatedness estimations within colonies, based on the microsatellite data. Standard errors were based on jackknifing over loci for within-colony estimates and over colonies for population estimates (Queller & Goodnight 1989). To calculate population allele frequencies, we weighted colonies equally in all analyses. We calculated 95% confidence intervals (C.I.) assuming that the jackknifed pseudovalues followed a t-distribution.

To estimate what proportions of males were produced by the queen and the workers we used a maximum likelihood method (modified version from Hastings et al. 1998, Arévalo et al. 1998). Under the assumption that we knew the queen, worker, and male genotypes at a locus we sought the maximum likelihood, $L$, of the parameter $Q$, the fraction of males drawn
from the queens. For each male-producing colony, we calculated

\[ L = K \prod_{\text{males}} \left( Q \prod_{\text{loci}} f_{qi} + (1 - Q) \prod_{\text{loci}} f_{wi} \right) \]

for values of \( Q \) between 0 and 1, which we sampled at intervals of 0.01. \( K \) is a multinomial constant that never has to be calculated because it multiplies all \( L \)'s by a constant, leaving comparisons among them unaffected. For each male allele considered in turn, \( f_{qi} \) and \( f_{wi} \) are the frequencies of that allele in the queen and in the workers. Table 1 shows the values taken by \( f_{qi} \) and \( f_{wi} \) for the four possible configurations of alleles at a locus when the queen is singly mated. In contrast to earlier papers (Arévalo et al. 1998, Hastings et al. 1998), we used the expected frequencies in workers rather than the observed frequencies in sampled workers. This is because our colonies had a rather simple structure, with one singly mated queen. Thus, if we sampled 15 AB workers and 5 AC workers, we still assumed that the laying worker pool consists of 50% of each type, because they must have come from an BC queen mated to an A male.
Table 1: \( f \) values for the likelihood function, \( L \).

<table>
<thead>
<tr>
<th>Worker genotypes</th>
<th>Inferred queen genotype</th>
<th>( f_{qi} ) if male allele matches queen</th>
<th>( f_{wi} ) if male allele matches worker</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>AA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AA,AB</td>
<td>AB</td>
<td>1/2</td>
<td>A 3/4, B1/4</td>
</tr>
<tr>
<td>AB,AC</td>
<td>AC</td>
<td>1/2</td>
<td>A 1/2, B1/4, C1/4</td>
</tr>
<tr>
<td>AB</td>
<td>AA or BB</td>
<td>1 or 0*</td>
<td>A 1/2, B1/2</td>
</tr>
</tbody>
</table>

* See text for combining these alternatives
In order to avoid damaging the colonies, we did not collect and genotype the queen. Instead, because the colonies are headed by one singly-mated queen, we could infer her genotype, and her mate's, from the worker genotypes. For the second and third cases in Table 1, this is straightforward. For case 1, in which all workers are homozygous AA, we assumed the queen is AA and her mate A, and we set $f_{qi} = 1$ (and also $f_{wi} = 1$) whenever a male has the A allele. Because we genotyped 7 to 11 workers per colony we neglected the chance that the queen is AB and transmitted no B alleles to the genotyped workers because the probability of missing an allele of a heterozygote queen when 7 daughters are genotyped is 0.008, low enough to be highly improbable. The more difficult case is the last one in Table 1, in which workers are all heterozygotes and we cannot distinguish between AA x B colonies from BB x A colonies. Whenever this happened, we calculated the colony likelihood, $L$, separately for each case, and then combined them (if two loci are ambiguous there are four separate cases to be combined). We combined them by weighting the separate $L$ estimates by their relative probabilities. If the frequencies of the two alleles are $p_A$ and $p_B$, then the probability of an AA x B mating is $p_A^2 p_B$ and of a BB x A is $p_A p_B^2$. The probability that it is AA x B over the summed probabilities of both
alternatives is $p_A/(p_A+p_B)$, and the corresponding value for BB x A is $p_B/(p_A+p_B)$. These are the weights used to combine the separate likelihoods. We calculated the L's separately across the whole colony before combining them because to treat each male separately would allow combinations that are not possible, if the males are queen produced, they must all be A or all be B, they cannot be a mixture of A and B

**Behavioral data**

The provisioning and oviposition process in *S. quadripunctata* *quadripunctata* is characterized by interactions with many body contacts between workers and the queen (Nogeira-Ferreira et al. 1998). When the queen finds an empty cell, ready for oviposition, on the edge of the horizontal comb, she "fixes" it (Sakagami et al.1965a) by staying very close to it and putting her front legs around the cell. Some workers move into a "hanging" vertical position on the outer edge of the comb, on the side of the empty cell, with their heads at the cell opening, facing the queen. Other workers collect on the horizontal comb around the queen. The queen's head often covers part or all of the cell. Workers hanging from the cell may dart and lunge at the queen. When a worker closely approaches the queen, the queen attempts to tap the worker with her antennae and front legs. Hanging
workers sometimes also put their head in the cell or dive partially into it during this preprovisioning period. Over time, their movements become shorter and more frequent. One of the workers then dives into the cell and discharges larval food into it, while the queen taps her side and back. Other workers then discharge into the cell. After several discharges, a trophic egg may be deposited on the inner rim of the cell. The queen inspects the cell, eats the worker egg if there is one, and often drinks some of the larval food. She subsequently deposits an egg herself on the top of the larval fluid, leaving the cell afterwards. One of the workers, often standing behind the queen before the queen lays her egg, will close the cell by putting her abdomen into the cell and performing rotational movements, pressing the cell rim inward with her mandibles while counterbalancing it with her abdomen inside the cell. During the last phase of cell closure the worker removes her abdomen from the cell and uses her mandibles to close the tiny hole, others often assist her.

We watched and analyzed a total of 92 POPs, (30 POPs before and 62 POPs during the period of male production). Definitions of the measured parameters are as follows:

(1) **Darting (DA)**: a hanging worker pushes her body forward, towards the queen and then moves away from her again. The head of the queen and the
workers come very close to each other, but do not necessarily touch. As soon as the worker and queen antennae touch, the queen moves back a little. Movements in this category vary in velocity from a slow to a rapid movement. We often observed that the tongue of the darting worker was stretched out and was inserted between the mandibles of the queen. However, we could not always confirm this because the dark tongues of the workers were hard to see against the dark empty cell. For this reason, every time we observed a worker with or without an outstretched tongue approaching the queen in a slower speed than lunge, we identified it as darting.

(2) **Lunging (LU):** a hanging worker pushes her body forward quickly and hits the head of the queen, pushing the queen away from the cell. This forward movement is followed by a rapid retreat. The worker often pushes the queen's head from underneath at an angle. Lunging is noticeably quicker and more energetic than darting and the worker often opens her wings in the forward movement. During lunging some workers extrude their tongues.

(3) **Inspection:** a worker darts forward, pushes the queen away if she blocks the cell and puts her head or a part of it into a cell. The queen taps the worker's back and the worker rapidly retreats.
(4) **Diving**: See inspection, except the worker inserts not just her head but also her thorax into the empty cell.

(5) **Discharging**: A diving worker contracts her abdomen so that her abdomen visibly shortens, regurgitating larval food into the cell.

(6) **Fixation interval**: The time between initial cell fixation by the queen and the first discharge by a worker into the cell.

(7) **Operculation interval**: The time interval during cell closure that the closing worker has her abdomen in the cell.

We also measured (8) the average number of workers hanging from the focal cell and (9) the number of workers standing within about 1.5 cm of the queen during POP.

Tapping of workers by the queen was excluded since it predictably happened every time a worker came close enough to the queen. Complex queen-worker interactions, such as hypnotic turning and hypnotic cell barricading (Drumond et al. 1996, Nogeira-Ferreira et al. 1998) were excluded from the analysis as well because they occurred in very low frequencies during both periods and nearly always before the queen really fixed the cell, which was before we started recording the various elements of the POP. To be able to compare different frequencies in the statistical
analysis, we calculated the relative frequencies of darts, lunges, inspections and dives by dividing their number in a POP by the fixation interval.

Though we did not measure ovarian development of workers that were involved in the POP, we know that some had developed ovaries because workers laid trophic eggs. We did not observe worker laying of non-trophic eggs (laid on the fluid rather than on the side of the cell). However, since cryptic worker reproduction often happens in stingless bees while workers close the cell (Beig 1972, Bego 1982, Koedam et al. 1999) we assumed that reproducing *S. quadripunctata quadripunctata* workers might apply that strategy as well. Workers might also return and lay eggs after the POP.

Our measured variables did not significantly differ from a normal distribution (Kolmogorov-Smirnof test $P>0.05$). For each behavioral variable we used a one factor, two-level incomplete block design within the procedure GLM (SAS Institute 1990). A multiple factor analysis gave similar results, showing that correlations among variables did not have important effects. The two levels in the analysis corresponded with the periods when males and no males were produced. The design was incomplete because some colonies were not observed both with and without males. Because the distribution of trophic worker eggs did not allow us to
include them in the GLM calculations we applied a G-test for those. For every test we used a significance level of $P<0.05$.

Assuming that POP involves conflict, we predicted that POP will be take longer and involve more conflict-like behavior during the period that males are produced compared to periods with just female production. Interactions that seemed agonistic, like darting and lunging between the workers and the queens, were expected to be more frequent during the male producing period compared to the period when no males where produced.

Though less obviously agonistic, we predicted that workers would inspect and dive into cells more frequently before discharging for checking out the cell they want to lay in. We also predicted that the worker interest in laying male eggs would mean that more of them will be involved in or near the POP, so as to have a hand in the outcome. But above all, we expected the operculation interval to be longer when workers have a chance to lay an egg in the cell they close (Table 2).
Results

Genetic data

Single mating

The workers within colonies were highly related to each other (Fig 1). On average, relatedness for all colonies was 0.75±0.04 (± 95% C.I.). The 95% confidence interval in all colonies overlapped with full sister relatedness, indicating that the queens were singly mated. Workers generally share one allele (paternal) and no more than two other alleles (maternal). There were a total of six non-matching genotypes of older workers. We checked and concluded that those individuals could not have been progeny of the queen, even if she was multiply mated, nor could they be daughters of an older deceased queen who was the mother of the current queen. Those non-matching workers must have been drifters from other colonies that were not used for the study. Therefore, we excluded them from further analysis.

Worker preference
Fig 1: relatedness values (±95% CI) between workers for 18 colonies of

Schwarziana quadripunctata quadripunctata
The relatedness among workers was higher than the relatedness of workers to queens (Fig 2). The paired difference test (Queller 1994) showed that there is a significant difference between them (0.28, 95% CI 0.11, \( P<0.001 \)). The above data indicate that on genetic grounds workers should prefer to produce the males.

**Who produces the males**

The relatedness of workers to males was 0.51, (CI 0.16), which is almost the same as the relatedness of workers to queens (0.48, CI 0.20, Fig 2). This is what we would expect if the queen produced the males. If workers produced the males, the worker-to-male relatedness of 0.51 ought to equal worker-to-worker relatedness, and it does not (paired difference 0.24, 95% CI 0.11, \( P<0.002 \)). This indicates that the workers are not producing the males.

Similarly, the results for the maximum likelihood method do not support male production by workers. The maximum of the likelihood estimate was 100% queen production of males for 15 colonies. Furthermore, the likelihood curves dropped off sharply, indicating anything besides queen production of most males was much less likely (Fig 3). In one colony the maximum was 90% queen production, but even here this was only about
Fig 2: Relatedness of workers to workers, workers to queens, workers to males and queens to males for 16 colonies of *Schwarziana quadripunctata quadripunctata*. 
Fig 3: The relative likelihoods for the fraction of males produced by the queen of *Schwarziana quadripunctata quadripunctata*. Each of 16 curves represents one colony. For each curve, all values were divided by the maximum likelihood for that colony, so that all values are now scaled to a maximum of one.
20% more likely than 100% queen production. Clearly, the queens produce all or nearly all of the males.

**Behavioral data**

Individual colonies differed from each other and colony effect was highly significant (p < 0.0005, df=5) for all parameters except diving frequency and operculation interval. Only two of the measured parameters differed significantly between the male producing and the female producing period, and only one, fixation interval, was highly significant (Fig 4). Both of these significant differences during male production (shorter fixation interval, fewer inspections) were in the opposite direction to the one predicted by the conflict theory (Fig. 4A, D). Also, against expectations, there were not significantly more workers around the queen or hanging from the cell during male production than when no males are produced (Fig 4B). Nor was the relative number of darts, lunges, or dives significantly different (fig 4C, D). Finally, the operculation interval was not significantly different between the two periods, providing no evidence of workers delaying closure in order to lay eggs (fig 4A).
Fig 4: The means±SE for the parameters fixation interval and operculation, the number of workers around the queen and the number of workers hanging from a cell, the frequency of inspection and dives in the cells and the frequency of darts and lunges during the POP in the period that no males were produce (empty bars) compared to the times of male production (hatched bars). In each case we expected the hatched bars to be higher.
We observed fewer trophic eggs laid by workers in the period with no male production (0.10 per cell) than in the period that males were produced (0.42 per cell, \( G \) test, \( G_1, P<0.05 \)). Therefore, in the male-producing period there were probably more workers with developed ovaries present that could have produced males than in the period when only females were produced. Further, observations revealed that hanging workers during the POP consisted of a pool of individuals replacing each other. This was especially obvious in the period of male production, hanging workers were pushing each other for space. The maximum number of hanging workers could have thus been determined by available space. We could often observe the queen fixed at a cell with open mandibles, also hanging workers were frequently seen with open mandibles.

Discussion

Despite the genetic conflict over male production between workers and their queen, in *S. quadripunctata quadripunctata* the queen produces the males. In spite of developed worker ovaries, the genetic conflict does not manifest itself in an increased measurable behavioral conflict between the two castes.
We found no real indication that the POP during male production was more aggressive than in the period when no males were produced. The failure of the simple relatedness predictions about collective worker interests raises two related questions. First, why do workers allow the queen to lay the male eggs? Second, why is there little evidence of increased conflict during male production?

*Why do workers not produce the males?*

Worker policing theory predicts that, other factors being equal, a collective worker preference is expected to favor the class of males to which the average worker is more highly related (Woyciechowski & Lomnicki 1987, Ratnieks 1988). In *S. quadripunctata quadripunctata* colonies, workers are more related to worker produced males (sons and nephews) than to queen produced males (brothers). Based on these results we expected, assuming equal costs and benefits of worker and queen reproduction, that workers would monopolize male production or at least produce a fraction of the males. The lack of worker reproduction is not a result of undeveloped ovaries, since workers in this species do lay trophic eggs that are eaten by the queen. Why do workers forego male production even though the theory predicts they should compete with the queen over parentage of male
progeny? The two main possibilities are that queens suppress worker reproduction or that workers do not reproduce because reproduction incurs high costs to the colony, reducing everyone’s fitness.

Despotic queens could conceivably control the workers either by physical force or by manipulation through signals. A *S. quadripunctata quadripunctata* colony would appear to contain too many workers for the queen to suppress (Ratnieks 1988), but rather few workers (9-30) participate in a given POP. The queen is larger, approximately twice the size of workers. However, her mouthparts are weak (Schwarz 1948, Camargo 1974) so queen control by biting of workers seems unlikely. Even if the queen could control workers during POP, one might expect workers to come back and lay eggs when the queen has gone elsewhere.

The other way queens might be able to suppress their workers is by pheromones (Fletcher & Ross 1985). However, such manipulation is thought to be difficult to sustain over evolutionary time as the workers would evolve to not respond (Keller & Nonacs 1993). Still, the queen might gain temporary control if she is able to repeatedly evolve new chemical manipulations at low cost (Foster et al. 2000). In addition, queen pheromones may perform multiple functions, some of which are not connected to queen suppression, so that workers need to remain sensitive to
them in order to perform optimally (Winston 1987). For example, it may be difficult for the workers to evolve to ignore queen pheromones when males are produced, if they convey honest information during the rest of the year (Foster et al. 2000).

If worker reproduction entails high costs to the colony, worker policing could be favored even when queens are singly mated (Kikuta and Kazuki 1999, Foster et al. 2000). However, in most stingless bee species workers do not eat eggs (Zucchi 1993), except when the queen fails to eat a trophic egg or when cells are broken down because of damage (personal obs). Even without policing, high costs could lead workers to individually refrain from attempting to reproduce (Cole 1986, Bourke 1988, Ratnieks & Reeve 1992, Pollock 1996).

Costs could occur in at least three different ways. First, colony performance might drop if reproducing workers consume too much food or neglect other colony tasks. Second, in a contest involving physical fighting, workers might injure or kill their queen (workers in stingless bees regularly kill virgin queens, Imperatriz Fonseca & Zucchi 1995). Worker production would suffer during the delay before a replacement queen becomes reproductive. A replacement queen also means that workers would also suffer a large relatedness loss for new queen production ($r=0.75$ to $0.375$).
So physically punishing the queen may not be a very good option, but this still doesn't explain why workers do not lay eggs when the queen is not present. A third cost, the cost of replacing female with male eggs, may help explain this (Ratnieks & Reeve 1992, Foster & Ratnieks 2001). If workers replacing queen-laid eggs are not able to distinguish between female and male eggs, they would be replacing many eggs destined to be workers and depleting the future work force.

*Why is POP not more aggressive during male production?*

The results of the behavioral data do not support the theoretical expectation that conflict between workers and queen would be higher during male production. One possibility is that such an effect might have been counteracted by an opposing effect of increased food during male production. If more workers have larval food in their stomachs, the oviposition process may become more efficient and thus shorter. If so, it still appears that the effects of conflict over male production are not very strong.

Another possible explanation is that aggressive conflict might actually be adaptive in both periods. Workers with developed ovaries may be a threat all the time. Some workers also have ovarian development in the period that no males are produced, so their egg laying should be prevented by the queen.
However, we now know one important fact that was unknown at the
time the prediction was made: workers in this species produce no males. The
lack of elevated agonistic interactions during male production could most
simply be explained by the fact that workers have lost or given up the
contest for male progeny in this species. It would be interesting to repeat this
study in a species where workers do produce males.

If there is no conflict in *S. quadripunctata quadripunctata*, why do
worker-queen interactions appear to be aggressive? Perhaps the interactions
between workers and the queen during the POP are a holdover from past
evolutionary conflict (Crespi 1992, Zucchi 1993). The aggressive POP
behaviors could have been retained as vestigial or non-functional traits.
However, the behaviors might also have acquired new functions. For
example, the queen's eating of non-viable trophic worker eggs (Sakagami
1982) could have derived from the eating viable worker eggs (Crespi 1992).
Similarly, the darting and lunging behaviors that precede egg laying might
now serve primarily for co-ordination between the workers who provision
and the queen who lays the eggs (Sakagami at al. 1965a, Sommeijer et al.
1984b, Zucchi 1993), but they may have originally evolved in the context of
conflict.
Male production in other species

Despite the fact that on relatedness grounds stingless bee workers should produce at least part of the males, in some species (*Frisoeomelitta varia, Trigona minangkabau*) workers have no ovarian development and are thus not able to produce eggs (Terada 1974, Cunha et al. 1986, Suka & Inoue 1993). Behavioral studies show that in some other species (*Tetragonisca angustula, Trigona pauperea pauperea, Tetragona dorsalis, Leurotrigona muelleri*) workers have developed ovaries but still do not produce males in queen-right colonies (Sommeijer et al. 1984a, Sakagami & Zucchi 1974, Inoue et al. 1999, Grosso et al. 2000). However, because oviposition might be very cryptic and overlooked by observers, or because workers might police each other and eat any eggs workers lay, the addition of genetic data is desirable. Our study of *S. quadripunctata quadripunctata* is the first one where genetic markers confirm behavioral observations that workers do not produce males.

insects appear to have singly mated queens (Owen & Plowright 1982, Bourke 1988, Boomsma & Ratnieks 1996, Arévalo et al. 1998, Walin et al. 1998, Foster et al. 2000, 2001, Strassmann 2001), and yet most social insects appear to have queen-produced males (Arévalo et al. 1998, Choe 1988, Foster et al. 2000, 2001, Bourke 1988, Walin et al. 1998). Thus, the prediction that singly mated species should have worker-produced males may fail quite broadly. It therefore becomes an important question to determine the cause, whether it involves fitness costs or queen suppression of workers. Comparative studies are needed between species where workers do and do not contribute to male production. Stingless bees provide species of both types and are thus an excellent group for further study.
CHAPTER V

Behavioral manifestations of genetic conflict between workers and queens in the stingless bee *Paratrigona subnuda*
Introduction

An important question in the study of social animals is how effectively group members cooperate (Maynard Smith & Szathmáry 1995, Queller and Strassmann 1998). Genetic relatedness among group members facilitates cooperation while differences in relatedness can cause conflict of interest within a group (Trivers and Hare 1976, Ratnieks 1988, Bourke and Franks 1995, Keller 1999). The haplodiploid genetic structure of social Hymenoptera make them particularly valuable for the study of cooperation and conflict. As a consequence of haplodiploidy, certain family members are highly related, which facilitates altruistic behavior (Hamilton 1964, 1972). But because colony members are asymmetrically related there is also potential for conflict within these societies (Ratnieks and Reeve 1992). Whether potential conflict becomes real conflict depends on how good colony members are at resolving their disharmony.

One important conflict is over the parentage of males. In multiply mated honeybees, workers are most related to their own sons, but least related to the sons of other workers, so they police each others’ reproduction, with the result that males are produced by the queen (Ratnieks
1989; Ratnieks and Visscher 1989). Stingless bees are an intriguing group for the study of this conflict because they are closely related and ecologically similar to honeybees, but their colonies are typically headed by one, singly mated queen (Camargo 1972, Contel and Kerr 1976, Machado et al. 1984, Olivera Campos 1990, Peters et al. 1999). Workers under such a situation are full sisters (r=0.75) and this high relatedness has consequences for male production. Workers that produce unfertilized haploid eggs that give rise to males are more related to their own sons (r=0.5) than to the sons of others, just as in honeybees. However, stingless bee workers are more closely related to their nephews (sisters’ sons r=0.375) than to their brothers (mother’s sons r=0.25). In stingless bees, therefore, workers should prefer their own and their sisters’ sons over queen-produced sons while the queen, of course, favors her own sons. This situation can lead to conflict between workers and their queen (Ratnieks 1988, Crespi 1992, Peters et al. 1999).

This potential conflict over male production can become real conflict if workers are capable of producing male eggs (Ratnieks and Reeve 1992). Workers in many stingless bee species have ovarian development during a period of their life and are able to produce haploid, male destined eggs (Sakagami 1982, Engels and Imperatriz Fonseca 1990, Zucchi 1993) suggesting a potential for real conflict (Peters et al. 1999). Nevertheless,
only a few studies have focused on worker production of males in stingless bees. Up to now, eleven stingless bee species are known to have worker reproduction, if we include species evaluated behaviorally (Sakagami et al. 1965, Beig 1972, Contel & Kerr 1976, Machado et al. 1984, Sommeijer et al. 1984, Sommeijer and van Buren 1992, da Cunha et al. 1986, van Benthem et al. 1995, Inoue and Roubik 1990, Inoue et al. 1999, Koedam et al. 1999, Drumond et al. 2000). Clearly the list is far from complete since very few of the several hundred species of stingless bees have been carefully studied. Moreover, we have insufficient information on whether workers can monopolize male production in species with worker reproduction (but see Contel and Kerr 1976, Machado et al. 1984, Koedam et al. 1999). Recently developed molecular techniques for analyzing relatedness and parentage should increase the number of species with worker reproduction and can help to assess the proportion of males produced by workers for those species (Queller et al. 1993, Queller and Goodnight 1989, Peters et al. 1999).

The potential genetic conflict could be settled without any actual behavioral conflict by reaching a peaceful settlement (Ratnieks and Reeve 1992). Either the queen or the workers could give up male production, or both might contribute peacefully to male production in a colony without negative interactions (Inoue et al. 1999).
When conflict over male production is not settled, it could manifest itself in behavioral hostility between the workers and the queen during the egg laying process (Peters et al. 1999). Stingless bees are mass provisioners and egg laying has to be synchronized between the workers and the queen. Egg laying is a complex, ritualized, and species-specific set of behaviors between workers and their queen, wherein the workers build and provision a cell before the queen lays an egg in it and leaves it to the workers to be closed (Sakagami 1982, Engels & Imperatriz-Fonseca 1990, Zucchi 1993). Before queen oviposition a worker sometimes lays a trophic, non-viable egg, which the queen consumes (Sakagami 1982, Zucchi 1993). Depending on the species, workers can construct between one and 100 cells for sequential oviposition (Zucchi 1993). The set of interactions involved in the former is often referred to as provisioning and ovipositioning process (POP) and that of the latter as integrated oviposition process (IOP).

In the dispute over male production the queen and the workers could utilize different strategies to win the competition. Queens could obstruct workers from reproducing by staying at the cell in species that have POP and by constantly patrolling over the comb in species with IOP (Zucchi 1993). When queens discover worker reproduction they could push workers from cells (Sommeijer et al. 1984b) and they could consume worker eggs
(Sommeijer et al. 1984b). Workers, on the other hand, could escape queen control in three ways. First, workers might lay an egg in the cell and close it immediately before the queen can get to it (Sommeijer et al. 1984, Koedam et al. 1999). Second, they can lay their reproductive eggs after queen oviposition, resulting in two or more eggs in the cell, then either closing the cell themselves (Beig 1972, van Benthem et al. 1995, Imperatriz Fonseca & Kleinert 1996) or allowing closing by another worker (Beig 1972). And third, workers could reopen closed cells and lay their eggs next to the queen’s egg (Imperatriz Fonseca & Kleinert 1998, Koedam et al. 1999).

Although workers do not eat queen eggs themselves, Beig (1972) showed that in the stingless bee, *Scaptotrigona postica*, male larvae hatch earlier from the egg and kill female larvae. It seems less likely, however, that a worker's male larva can regularly destroy the queen's male larva.

We studied the outcome of conflict over male production in *Paratrechina subnuda*, a species that has singly mated queens (Peters et al. 1999) and an integrated oviposition process. Workers in this species build 8-33 cells at a time and then the queen oviposits in them in the same IOP (Zucchi et al. 1997). Workers have ovarian development and have been seen laying trophic eggs (Zucchi et al. 1997), but actual worker reproduction has not been verified. The aim of the study was threefold. First, to verify
whether there is a potential conflict between workers and queens, we reconfirmed the mating status of *Paratrigna subnuda* queens for six colonies. Second, we sought to determine if workers or the queen produced the males, or in what proportions they divided the male production. Third, we sought to assess possible behavioral conflict over male production and to determine its relationship to genetic conflict.

**Methods**

*Species and study area*

*Paratrigna subnuda* is a common stingless bee in the Brazilian Atlantic rain forest. It nests 25 to 120 cm under ground (Schwarz 1942, Imperatriz-Fonseca 1972). The nests are spherical and are protected by several sheets of involucrum. Inside these sheets is a helicoidal brood comb with small, egg-shaped honey and pollen pots around it (Imperatriz-Fonseca 1972, 1976). This species is very suitable for behavioral study because new combs are built on top of older combs, facilitating behavioral observation of the oviposition process. The size difference between workers and queens is pronounced. Not only does the queen have a larger head and thorax (Imperatriz Fonseca 1976), but physogastric queens (1.2-1.8 cm) are at least
twice as big as workers (0.5-0.8 cm) and can push workers away relatively easily.

The experiment was carried out at the University of São Paulo (Brazil) between March and December 1999. We used six colonies of *Paratrigona subnuda*, which we dug up in the Atlantic Rain forest near Cotia (46°56’ W, 23°39’ S), Brazil. After excavation we transported the colonies to the bee laboratory in São Paulo, removed the involucrum layers on the upper side of the colonies, and transferred the nests into observation hives. The observation hives consisted of wooden boxes covered with a glass lid. We placed these hives into a thermo box with a glass lid that kept the colonies at a constant temperature of 28°C (Sakagami 1966). When the colonies were not used the thermo boxes were covered with thick black fabric and styrofoam plates. All colonies had an exit tube leading outside, so that the bees could forage normally.

All the colonies we studied were similarly sized and were producing males. Colonies contained one laying queen, 3000-4500 workers, 150-400 males, a few virgin queens, and six to eight layers of brood comb.
Genetic data

In all we genotyped 107 workers (10 to 34 per colony) and 418 males (from 27 to 108 per colony). We collected two or three batches of males with three-week intervals over a period of two months. We genotyped workers and males at five moderately polymorphic microsatellite loci: Mbi232AAG, Mbi233AAG, Mbi254AAG, Mbi259AAG, Mbi278AAG (Peters et al. 1998). These loci had from two to eight alleles in our population. We used workers and males too young to have left their natal colony. We identified these individuals by their lighter pigmentation and their often bent-down wing tips. We extracted DNA, set up polymerase chain reactions, and visualized PCR products on polyacrylamide gels following the protocols of Strassmann et al. (1996). To estimate relatedness within colonies for our microsatellite data we used the computer program Relatedness 5.07 for Macintosh (Goodnight and Queller 2000; available at http://Gsoft.smu.edue/Gsoft.html). Standard errors were based on jackknifing over loci for within-colony estimates and over colonies for population estimates (Queller & Goodnight 1989). To calculate population allele frequencies, we weighted colonies equally in all analyses. We calculated 95% confidence intervals (C.I.) assuming that the jackknifed pseudovalues followed a t-distribution.
To estimate the proportion of males that were produced by the queen or the workers we used a modified version of the maximum likelihood method from Arévalo et al. (1998). We have a set of worker and male genotypes, and we infer queen genotypes from the workers. The likelihood, $L$, for any value of the parameter $Q$, the fraction of males drawn from the queens is calculated as $L = K \prod_{\text{males}} \left( Q \prod_{\text{loci}} f_{qi} + (1 - Q) \prod_{\text{loci}} f_{wi} \right)$. $K$ is a multinomial constant that never has to be calculated because it multiplies all $L$'s by a constant. For each male allele considered in turn, $f_{qi}$ and $f_{wi}$ are the frequencies of that allele in the queen and in the workers. We calculate $L$ for all values of $Q$ between 0 and 1 at increments of 0.01 (further details in Tóth et al. in prep).

**Behavioral data**

We videotaped a total of 37 integrated ovipositions (IOPs) which included provisioning and laying in 536 cells. We started the analysis of the videotapes from two minutes before general arousal of the workers that precedes IOP and we stopped the analysis 3 to 22 minutes after the last cell that was laid in by the queen was closed by workers. In this way we could observe, should workers reproduce, if worker oviposition happened before queen oviposition or after, when workers closed cells or when cells were
already closed and workers had to reopen them to lay their eggs. In order to determine the effectiveness of queen disturbance, for every IOP we counted the total number of cells built and determined the frequency at which queens disturbed workers that were involved in the IOP. To estimate worker reproduction behaviorally, we calculated the proportion of cells reopened later by reproducing workers. We included only 32 IOPs (435 cells) in the analysis, and excluded IOPs where we stopped recording less than five minutes after the last cell was laid in, because cell reopening might have been missed if fewer than 5 minutes after laying were watched. We noted whether, while patrolling, the queen walked by a worker-opened cell and whether she was aware of the worker’s laying activity. We also recorded the worker’s reactions to the queen’s presence. For cells that were older and were opened at times other than right after an IOP, we tried to when they were built and laid in from their position from the edge of the brood comb. We characterized worker laying and worker-queen interactions for these cells as well.
Results

Genetic data

As expected, workers in the colonies were full sisters (pooled $R=0.74$, 95% CI±0.052, $N=107$). In each colony, all worker genotypes were consistent with a full sister group. Workers contributed substantially to male production. Depending on the colony, workers produced from 20% to 85% of all males (Fig 1). The average of the colony maximum likelihoods was 63.7% (± 17.1 s.d.). The likelihood curves were steep enough to conclude that male production was shared. In no colony was there a significant likelihood that the queen produced all the males. In only one colony was there a reasonable chance that workers produced all the males, but even in this colony this hypothesis was only one quarter as likely as shared male production.

When we looked at the samples separated in time by at least three weeks, the range of worker-produced males was broader, from almost none to all males produced by workers (Fig 2). We could not detect any seasonal or order effect in the fraction of males produced by workers at different times (Fig 2). In four colonies the proportion of males produced by workers did not substantially differ over time, but in two colonies they did, as
Fig 1: The likelihood for the fraction of males produced by the queen for six colonies of *Paratrigona subnuda*. In order to standardize the maximum likelihood to unity, the likelihood is presented as a ratio, with the maximum likelihood in the denominator.
Fig 2: The maximum likelihood for the fraction of queen-produced males for the individual colonies, at sampling periods three weeks apart. The error bars represent likelihood values that are 10% as likely as the maximum. Samples shown with an open, shaded and striped columns were taken with three-week intervals. Samples for colony 1 were taken in a different part of the year, but all the samples from other colonies were taken in the same time period.
indicated by little overlap of the likelihood curves from different time samples (Fig 2).

*Behavioral data*

Our video-recordings showed workers building and the queen ovipositing in, on average, 14.5 cells per IOP (SD=4.9, range: 7-27, N=37 IOPs). The actual number of cells must have been somewhat higher though, because sometimes the involucrum covered a part of the comb that we videotaped and sometimes the comb was too big to get analyzable footage of the whole. The number of cells/IOP in our colonies was thus similar to that observed in other studies (Zucchi et al. 1997, range 10-35). Colonies did not differ in the number of cells built per IOP (Kruskal-Wallis, P=0.91, df=5), so we pooled colony values for further analyses. On average, before queen oviposition workers laid 0.24 (SD=0.19, range 0-2) trophic eggs per cell. The queens ate all those eggs and then laid their own eggs. No reproductive worker eggs were laid before queen ovipositions.

As is typical for species that have integrated ovipositions, the queen kept patrolling over the comb after she oviposited. Workers that closed cells were easily displaced by the queen, or by another worker. Queens interrupted closing workers 2.8 times on average (SD= 2.1, range=0-7).
When the cell became unobstructed again the same or another worker resumed cell closing. On average 2.1 workers were necessary to close each cell (SD=1.7, range=1-5). None of the workers that closed the cells laid a reproductive egg in them.

Reproducing workers laid their eggs exclusively by reopening closed cells. Laying workers reopened one third of the cells that were provisioned in the most recent IOP (31.2%, SD22.3, N=32 IOPs, Fig 3), shortly after they were closed (average=80.3 s after cell closure, SD=74.3, N=134 cells). They needed on average just 45 s (SD=26) to open the cell, lay an egg and reclose the cell. Although we did not mark workers in the colonies, it seemed that laying workers were not involved in the IOP because laying workers came from underneath the comb right before they started to open the cell. Laying workers acted individually; other workers did not orient towards their activity.

While patrolling over the comb, the queen walked over the opened cells in 46% of the cases (Fig 4). The queen might not have been aware of all the cases in which cells were reopened. In only 28% of the cases did she pause and antennate the opened cell. When the queen walked along the cell and touched or pushed the worker with her body, 54.8% of the workers gave way (Fig 4), but right after the queen left workers resumed their activity.
again. Reproducing workers made a very small hole on the cells (approximate diameter=0.5 mm) that neither workers nor the queen could put their head or abdomen in. Laying workers just dropped an egg through the hole. In three cases we saw the queen trying to lay an egg in the opened cells, without success. Perhaps the hole of the cell was too small for the queen egg or perhaps the queen’s abdomen needs to be in the cell to lay an egg.

We found no evidence for effective queen control of worker reproduction. The queen did not frequent cells that were reopened by workers more often than cells that were not (t-test, P=0.96, t=-0.05, DF=433), suggesting that she did not know which cells would be reopened. There was no correlation between the proportion of workers backing off the queen while she was patrolling over the comb during an IOP and the proportion of cells reopened by workers right after the IOP (rho=0.01, P=0.97, N=32 IOP), suggesting that patrolling did not elevate queen control. Likewise, there was no correlation between the proportion of time the queen spent on the comb’s edge where the new cells were, and the proportion of cells reopened by workers (rho=-0.16, P=0.38), indicating that queen presence did not increase her control. We also did not find any correlation
Fig 3: Proportion of cells opened by workers during different IOP's pooled in six colonies
Fig 4: The frequencies of the queen walking by an opened cell, queen inspection of open cells and reproductive workers giving way to the queen pooled over six colonies.
between the number of cells per IOP and the proportion of cells reopened (rho=-0.10, P= 0.59). This indicates that the queen was not able to control worker reproduction more when there were fewer cells to be laid in than when there were more. When we looked at each colony separately, no significant correlations were found for the above effects. Similarly, individual colonies did not show a correlation between queens presence and the proportion of cells reopened, nor for the number of cells involved in the IOP and the proportion of cells reopened (Table 1).

Surprisingly, older cells away from the comb’s edge were also reopened and laid in by workers. These had been filled 7-15 IOP’s previously, which we estimate to be a period of about 1 or 2 days. Unlike the reproducing workers laying just after an IOP, these ovipositing workers were surrounded by a court of workers consisting of 6 to 12 individuals for the period the cell was open. The attending workers did not hinder the egg layers in their work. The average duration, from nibbling on a cell until a new egg was laid and the cell was closed, was 76.8s (SD=37.4 N=60). This was longer than the duration for cells that have been reopened just after the IOP (t=6.26 P<0.0001, DF=19). The queen was less likely to walk by a reopened cell outside the IOP than by reopened cells that were a part of the IOP (G=10.3, P=0.0013, DF=192). Often when the queen came close to the
Table 1: Spearman rank correlations between proportion of cells opened right after the IOP with three other measures: (1) the proportion of workers backing off from the queen (2) the proportion time the queen spent on the comb edge during an IOP and (3) the number of cells built for an IOP.

<table>
<thead>
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<th>Colony id</th>
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<td>0.98</td>
</tr>
</tbody>
</table>
laying worker the workers that were around the reproductive worker darted towards the queen.

We did not observe other workers reopening cells, which were worker laid, to oviposit them during the observation periods (3-22 minutes).

Discussion

This study shows that *Paratrigona subnuda* queens are singly mated and that there is an unsettled conflict between workers and their queen over who should produce the males in the colonies. As Peters et al. (1999) suggested, signs of unresolved conflict include worker contribution to male production and behavioral clues such as queen consumption of worker eggs and antagonistic interactions between the two castes. We argue that the conflict in *Paratrigona subnuda* must be unsettled for several reasons. In the genetic part of the study we found that despite similar colony sizes and resources workers contributed substantially to male production in some colonies and less in others. Also, the fraction of males produced by workers over time in single colonies was not constant in all colonies, which again indicates that
neither of the parties won the conflict. Our behavioral observations also indicate an ongoing conflict. Overt egg-eating conflict does not appear to be supported by our observations since the queen ate only trophic eggs and not reproductive worker eggs. However, we observed other signs of behavioral conflict between workers and queens. A peaceful division of reproduction might be achieved by workers laying and closing the cells before the queen laid her egg. Instead, workers reopened queen laid cells, and reoviposited, presumably resulting in the destruction of the queen’s progeny. When workers were about to lay their eggs, the queen sometimes approached and tried to push them from the cell, and 45% of the workers resisted the queen’s pushing and went on with egg laying. Even when the queen was able to push laying workers aside, the workers came back to the cell to finish egg-laying after the queen left. Furthermore, workers made the holes in the cells too small and the queen seemed unable to lay her eggs in reopened cells.

Non-reproducing workers collectively seemed to agree with their reproductive sisters, since reproducing workers were not hindered by other, non-reproducing sisters. This was especially visible when workers opened older cells, because these workers had a rosette of 6-12 other workers grouped around them. We never observed reopening a cell where a worker had laid an egg, although the time interval we observed was brief (3-22
minutes). Workers in other stingless bees sometimes compete not only with the queen but also with each other for male production. In *Friesella schrottkyi*, *Melipona quadrispectata*, and *Scaptotrigona postica*, some cells have up to four eggs (Beig 1972, Sakagami et al. 1965).

We did not find evidence that the queen was able to influence worker reproduction, as measured by the fraction of cells reopened. This was uncorrelated with the number of workers backing off from the queen in an IOP, perhaps because the queen encountered more workers simply when more workers were on the upper comb. The proportion of cells reopened also did not correlate with the total number of cells oviposited in the IOP. Any queen control might be expected to be stronger with fewer cells than many cells. Cell reopening was also uncorrelated with the amount of time the queen spent on the comb edge, where the new cells were. This suggests that workers did not require the absence of the queen at a cell to lay their male eggs. The above arguments do not entirely exclude queen control during the IOP, because reproducing workers were most probably not involved in the oviposition process but instead laid their eggs after the IOP. Despite this, our direct observations showed that the queen could not control laying workers because when workers were reproducing and the queen was aware of it the queen could never prevent them doing so.
Knowing that the queen did not have control over worker reproduction and that other workers agreed with their reproducing sisters, it seems strange that workers did not dominate male production completely. Workers are unlikely to be able to distinguish between male and female eggs laid by the queen (Nonacs and Carlin 1990) so replacing queen-laid eggs may be costly in terms of the number of workers raised. Even if stingless bee workers could distinguish male and female eggs, several observations suggest that workers laid an egg in a cell regardless of whether it contained a male destined or female destined egg. First, reproducing workers that were not involved in the IOP could not know the sex of queen-laid eggs because the cells were closed when laying workers arrived. Second, we never observed a worker opening a cell and closing it without laying an egg in it. Furthermore, workers usually opened a cell very close to the place they entered the comb and they never walked around on the comb antennating cells before they started their opening activity. If workers could not know which sex of egg they were replacing, they might have restricted their reproduction in order not to destroy too many worker eggs.

Worker laying after the IOP, rather than during it, seems beneficial for the reproductive workers, both in terms of their own reproduction, and in terms of minimizing disruption of colony function. First, in this way workers
can evade the physical pushing with the heavier and bigger queen. By waiting until the cells were closed and then reopening them just a little bit, the workers seem to prevent the queen from sticking her head into the cells and eating reproductive worker eggs, and perhaps also prevent the queen from laying another egg in the opened cell. Second, other workers did not disturb reproductive workers in their activity. Right before and after provisioning, when there is a general arousal of workers on the upper comb, workers that are involved in the IOP are in an excited stage and run over the comb bumping into each other. Laying workers could be disturbed too much by the runners. Third, workers laying after the IOP could minimize disruption. During the IOP the queen might be more aware of what is going on and could pause longer at individual cells and consequently might not get to every cell to lay her eggs, reducing the efficiency of the IOP. Cells that are not oviposited in within a certain period are cleaned and broken down again. Worker oviposition right after the IOP, when cells are already closed, might keep the colony more efficient.

Why workers laid their eggs not only in freshly closed cells but also in older ones is more difficult to explain. Laying in older cells might be less effective if queen eggs hatch earlier than worker eggs, and queen’s larvae kill or eat the worker eggs. However, it is not known whether queen-
produced larvae also kill larvae in the cell or whether it is just a feature of worker-produced male larvae. Perhaps, while workers are not able to detect the sex of the eggs, they can detect the sex of the larva (even through the cell cover) and noticed that the cell contained a male larva. If queen-produced bees do not kill other cell occupants, it may still not too late for workers to reproduce.

Reduced queen presence at old cells cannot explain worker laying in these cells because our results showed that the queen was not able to influence worker reproduction. Maybe laying workers had only a short time to lay their eggs on the comb’s edge since shortly after the IOP the building of new cells began and comb edge was occupied by building workers making it impossible for others to lay. This hypothesis is supported by our data, since most worker eggs laid in older cells were laid before or several minutes after the IOP. However, laying workers might be able to reopen cells closer to the comb’s edge and would not need to open cells that far away from it. Though not very likely, it could also be that older cells opened by workers contained dead larvae. Workers opened and laid eggs in older cells that were not far from each other. It was usually a region where the comb was bending upward such that it would not touch the comb underneath it, the cells that were reopened were mostly supporting the rest of the
growing comb. Due to friction larvae in those cells could have died. For this to be true, *P. subnuda* would have to be an exception to the rule that stingless bee workers break cells down if larvae die instead of laying an egg into the cell. On hygienic grounds, it seems unlikely that they would not remove a dead larva.

This study showed that *P. subnuda* workers employ some unusual strategies in competing with the queen over male production. They re-open closed cells, but not completely, making tiny apertures. In addition, some workers open and lay in cells provisioned during IOPs that occurred some time previously. More detailed studies will be required to assess the relative importance of worker laying immediately after the IOP compared to laying in older cells. Because we videotaped colonies shortly before IOP to approximately 20 minutes after it finished we could have missed a substantial part of later laying. Furthermore, we do not know which worker-laid eggs succeeded at producing a male. Nevertheless, it is clear that the worker strategies as a whole are fairly effective ways to compete with the queen over male production. The queen seems powerless to stop worker laying and workers contribute a majority of the male eggs. However, despite her apparent lack of power, the queen also gains a share of the male
production, perhaps because eggs giving rise to males are indistinguishable from those that will produce workers.
CHAPTER VI

Do agonistic worker-queen interactions predict conflict between the castes in stingless bees?
Introduction

Conflict is an elemental feature of social organisms (Maynard-Smith & Szathmáry 1995, Keller 1999). A potential for conflict in insect societies exists among their group members over how resources should be invested into the production of offspring (Ratnieks & Reeve 1992, Bourke & Franks 1995, Queller & Strassmann 1998). How conflicts are resolved is essential for our understanding of the evolution of sociality (Keller 1995).

Social Hymenoptera are ideal for the study of conflict because of their haplo-diploid sex determination (Trivers & Hare 1976). A mated female has control over fertilization of her eggs: fertilized eggs become females, unfertilized eggs produce males. This creates asymmetries in relatedness within colonies, resulting in potential conflict among female colony members over who should produce the males (Ratnieks & Reeve 1992, Strassmann & Queller 1998). Although in many social insects, workers are not able to mate and thus cannot produce females, they can have male progeny by laying unfertilized eggs. Females are most related to their own sons (r=1/2) and on relatedness grounds thus a female would prefer her own sons. Who the opponents in the conflict will be depends, however, on the mating status of the queen. If the queen mates multiply, workers are more
related to their brothers (queen produced males $r=1/4$), than to their nephews
(worker produced males $r$ close to $1/8$), which results in workers policing
each other, and allowing the queen to produce the males (Woyciechowsky &
Lomnicki 1987, Ratnieks 1988, Ratnieks & Visscher 1996). When the
colony has a single, once-mated queen, workers are more related to their
nephews ($r=3/8$) than to their brothers ($r=1/4$) and a worker-queen conflict
over male production is predicted (Ratnieks & Reeve 1992, Strassmann &
Queller 1998). The outcome of the conflict however, does not depend only
on relatedness but also on costs and benefits (Hamilton 1964, 1972). Queen
manipulation might play a role as well (Foster et al. 2000).

Most stingless bees have a single once-mated queen, making them
interesting for studying worker-queen conflict (Peters et al 1999). Moreover,
stingless bees form a species rich taxon, with more than 400 species
(Michener 1974); this makes comparative studies very appealing. Although
all species have similar general ecology, they vary in nest architecture,
individual activity level, size of their individuals and the number of
individuals forming a colony. Because of these differences across species,
potential conflict over male production could be resolved differently
depending on the species. In almost all species worker-queen interactions
seem to be agonistic, though in some species agonistic interactions are more
intense and occur in higher frequency than in others (Zucchi 1993). So far from genetic and behavioral studies we have indications that workers do not equally contribute to male production in all species (Sakagami & Zucchi 1974, Sommeijer et al. 1984a, Suka & Inoue 1993, Inoue et al. 1999, Chapter 4). Genetic studies are important to determine with certainty who in the colonies is producing the males. However, in order to confirm whether the conflict is resolved or not, behavioral analysis of worker-queen interactions is desirable.

Stingless bees mass-provision their cells, meaning that the workers have to regurgitate larval food into the new cells before the queen can lay an egg in them (Sakagami 1982, Engels & Imperatriz Fonseca 1990). Thus mass provisioning requires both the queen and some workers to be present at the new cells. Moreover, to coordinate the timing of the oviposition properly both castes have to interact with each other (Sakagami 1982). New cells can be built and oviposited one-by-one, or in groups depending on the species. Usually, when a new cell is ready a group of workers and the queen collect around it, with the workers and the queen on opposing sides of the cell. Both castes inspect the cell repeatedly; workers however have a tendency to avoid the queen by withdrawing if they get too close to her, the queen in turn tries to touch workers with her antennae and forelegs (Zucchi 1993). During this
phase, workers often dart or even lunge at the queen. When excitement is at its highest, one worker regurgitates into the cell, followed by other bees provisioning the cell as well. Workers often quickly retreat or jump back after regurgitation. In other species when the cell has enough larval food in it the queen lays her egg on the fluid and one of the workers closes the cell (Zucchi 1993). When many cells are built and have to be provisioned at the same time the queen does not stay at one cell the entire time, but patrols over the comb visiting the different cells. When agitation is at its highest workers almost synchronously provision the cells. The queen goes then to the individual cells to oviposit in them (Zucchi 1993).

Although the queen patrols between the different cells when many cells are made and is not stationary, opposed to in species where just one cell needs to be oviposited, general queen-worker interactions are markedly similar in both groups (Zucchi et al. 1999). Although workers often show avoidance towards the queen when very close to her, they also seem to resist the queen’s acts and even molest the queen. The queen’s behavior can range from pushing workers out of her way to violently tapping their backs. Antagonistic behaviors between workers and queen can thus be categorized into queen dominance of workers, worker avoidance of the queen and worker resistance/aggression to queen’s presence.
In this study we investigated whether it is possible to explain the oviposition process in stingless bees in terms of conflict resulting from relatedness asymmetries. We examined eleven species with known worker contribution to male production and investigated whether the level of behavioral aggressivity is correlated with the proportion of worker produced males in the different species.

**Methods**

*Species characteristics*

For this study we compared worker-queen interactions during the oviposition process in eleven stingless bee species. All the species have a Neotropical distribution and a single queen. The behavioral analysis of the oviposition of these species is based on own observations and studies of others: *Melipona quadrispilota* (own observations, Sakagami et al. 1965) *M. marginata* (own observations), *Scaptotrigona postica* (Sakagami & Zucchi 1963), *Tetragonula clavipes* (Sakagami & Zucchi 1967), *Frisella schrottkyi* (Sakagami et al. 1973), *Paratrigona subnuda* (own observations, Zucchi et al. 1997), *Schwarziana quadripunctata* (own observations,
Nogeira Ferreira et al. 1998), *Eurotrigona mulleri* (Sakagami & Zucchi 1974) and *Trigona nigra pauperea* (Sommeijer et al. 1984). Worker reproduction was confirmed genetically (Chapter 3, 4 &5, Machado et al. 1984) for most species except for *Friesella schrottkyi*, *Trigona nigra pauperea* and *Eurotrigona muelleri* for which behavioral and cytological observations provide the reproductive status of workers (Sommeijer et al. 1984, Imperatriz Fonseca 1998, Sakagami and Zucchi 1974).

**Analysis behavioral observations**

We classified behaviors occurring during the oviposition stage that indicated worker-queen conflict (Table 1). These behaviors were categorized according to their nature into 1) queen avoidance by the workers, 2) worker aggressivity towards the queen or 3) queen dominance towards the workers. Workers may avoid the queen every time they meet her or just in certain occasions. Workers can also actively push the queen away or hit the queen with their head. The queen in turn can push workers aside and tap on their backs with her antennae and fore legs. We tabulated queen-worker interactions for each species giving a 1 if it occurred and a 0 if it did not occur in the species. We then summed the value of all interactions per species per category as well as all the categories. We performed the
Spearman rank correlation test on the amount of worker reproduction versus the amount of aggression between workers and queens in different species.

Results

The amount of queen-worker aggression during the oviposition period was differed between species (Table 1). While in some species there are few agonistic interactions between the workers and the queen, other species showed twice as many agonistic interactions. Worker behavior towards queen did not have a significantly different score level than queen behaviors towards the workers (t=0.9, P=0.39, DF=11). Worker avoidance of the queen was not correlated with queen dominance (Spearman, rho=0.41, p=0.17). Also, the level of agonistic worker behaviors towards the queen did not correlate with queen dominance (Spearman, rho=0.47, p=0.12). The level of agonistic interactions is not significantly correlated to the amount of worker reproduction within species (Spearman, rho=0.15, p=0.61, Fig1).
Table 1: Queen-worker interactions that indicate conflict between the castes and the amount of worker reproduction per species

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<th>Category 3</th>
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</table>
Fig 1: The summed worker-queen interactions indicating conflict for species where workers substantially, little or do not contribute to male production
Discussion

Our results suggest that queen-worker interactions might be more rigid than we thought. Although the ‘aggressivity’ level between workers and queens differed per species, aggressivity was not correlated to the amount of worker reproduction in different species.

Our results, however, could have been affected by several factors. First, behaviors we defined as aggressive might not serve aggressive purposes. Second, if the behaviors are indeed aggressive, they may represent past aggression and possess no adaptive value at the present. Third, we gave each interaction the same value despite the fact that some seemed more aggressive than others. Fourth, we do not know whether all species we analyzed were only producing females with no potential queen-worker conflict at the time or whether they were in their male production period. Inconsistency in the period when observations were made could have resulted in partially inconsistent outcomes.

Perhaps, interactions that seem aggressive to us have different communicative values to the bees. Since the nest is normally dark, all the communication between workers and queens happens in darkness. Instead of being aggressive, darting and lunging workers might just check whether the
queen is still at the cell, as well as communicate to the queen that they are there. The frequency of darting and lunging toward the queen might indicate the number of workers and their readiness to go to the next phase. Timing in stingless bees is important because the queen has to be present and lay her egg not too long after the cell is provisioned. Likewise, the explanation for workers retreating from a cell after insertion and provisioning may not be queen avoidance, but rather workers might just make room for other workers to get to the cell. In extreme cases workers jump away from the cells after provisioning, however, workers also do that when the queen is not present, so they might do that for other reasons than avoiding the queen. Also, queen behaviors such as staying close to the cell and tapping workers’ backs can be explained as a different form of communication than aggression. By staying close to a cell, the queen might indicate to the workers that she is close to lay an egg. By tapping workers’ backs the queen might stimulate them to provision the cell. However, workers leaving the cell at queen’s arrival, as in Friesella schrottkyi, or the queen pushing workers from cells and workers’ resistance to that, would be difficult to explain except in the terms of conflict. Also general worker behavior, which is characterized by queen-avoidance when she is too close to workers suggests a certain amount of conflict.
It could be also, that queen-worker interactions were aggressive in the past, but that they received a different communication value later on in evolutionary time and so they do not represent aggressivity at the present. The meaning of behaviors between the workers and the queen might have changed and aggressive looking behaviors could represent a different motive (Crespi 1992)

Even if most the interactions we analyzed were aggressive, it was difficult to represent them in a quantitative way. Some behaviors occurred with a higher frequency in certain species than in others. In our analysis, however we gave a value to a behavior if it occurred in a species, and did not give gradation in frequencies. To quantify worker-queen interaction is difficult if not impossible, because there are not only differences between species but also within species according to colony conditions.

Although our study on *Schwarziana quadripunctata* (see chapter 4) showed that there is no elevated aggression between workers and queens during male production, in other species that might not be true. However, there might be an elevated level of aggression in species where workers did not give up reproduction. In this study, we did not exclusively analyze worker-queen interaction during the male production period, neither do we know in what phase colonies described by others were. An analysis of all
species while they were in their male producing period might have affected
the results, although probably not in a significant way. The total aggressivity
score in *Schwarziana quadripunctata* and *Tetragonisca angustula* is high,
but since workers do not produce males it should not be higher than in
species were workers do reproduce.

Although the above-mentioned explanations for our results are valid,
only the first two could give a valid solution for our data. It is difficult to
decide which of these two possibilities might be more valid, partially
because the explanations are not exclusive. To get a better idea which
explanation fits stingless bees better more species need to be involved in the
analysis, and thus more species need to be studied.
CHAPTER VII

Worker reproduction in stingless bees
Introduction

Sociality in Hymenoptera has evolved and persisted independently maybe a dozen of times (Carpenter 1953, Wilson 1971). Insect societies are very successful, since their high degree of cooperation allows them to ecologically dominate terrestrial systems (Wilson 1971). Social organisms, however, are predisposed to internal conflicts as well and these conflicts potentially weaken the forces that hold colonies together (Trivers & Hare 1976, Bourke & Franks 1995, Queller & Strassmann 1998). How insect societies remain intact despite the destructive effects of conflict and how contests are settled is an important question (Keller 1995).

Hymenoptera have a peculiar genetic asymmetry due to their haplodiploid method of sex determination. Usually, fertilized eggs become females and unfertilized eggs become haploid males. A mated queen has control over which type of egg she lays. Haplo-diploidy results typically in high relatedness among females (Hamilton 1964). High relatedness among working colony members favors cooperation (Hamilton 1964, 1972). Yet, relatedness asymmetries can also cause disharmony within colonies (Ratnieks & Reeve 1992, Strassmann & Queller 1998). Since females are not equally related to male progeny, conflicts can arise over who should
produce the males. Females are related to their own sons by 1/2, but they are less related to other females’ sons. In insects that have a multiply mated queen, such as honeybees, workers are on average less related to the sons of other workers (r close to 1/8) than to sons of their mother, the queen (r=1/4). Consequently, honeybee workers prevent each other from reproducing, whereas they unanimously allow their mother, the queen, to produce males (Woyciechowsky & Lomnicki 1987, Ratnieks 1988, Ratnieks & Visscher 1986). However, in species where the queen mates singly, such as in stingless bees (Peters et al. 1999), workers are more related to their sisters’ sons (r=3/8) than they are to their mother’s sons (r=1/4). Hence, on relatedness grounds workers should allow each other to reproduce and collectively oppose queen production of males (Queller and Strassmann 1998). In contrast to honeybees where most of the workers have degenerated ovaries and worker reproduction is rare, workers with ovarian development are very common in stingless bees (Engels & Imperatriz Fonseca 1999).

Data on worker reproduction are, however, difficult to obtain because workers produce both trophic eggs that lack nuclei to feed the queen and reproductive eggs that develop into males (Sakagami 1982). So observation of worker ovarian development or egg production is usually not sufficient to infer worker reproduction. In stingless bees larvae are mass provisioned
(Sakagami 1982). The cells have to be provisioned with larval food before the queen can lay an egg in them. Then the cells are closed a worker that pushes the cell rim inward with her legs and mandibles while pressing it against her abdomen that is in the cell (Engels & Imperatriz Fonseca 1990). Reproductive workers can lay an egg before the queen oviposition and close the cell immediately or after the queen egg is laid, yielding at least two eggs in the cell (Beig 1972, Bego 1982, Imperatriz-Fonseca & Matos-Kleinert 1998, Inue et al. 1999). When a female egg by the queen and a worker’s male egg are in the same cell the male larva hatches first and kills the female one (shown for Scaptotrigona postica and Melipona quadrijasciata anthoides, Beig 1972).

Previous research has indicated that despite single mating of stingless bee queens (Silva et al. 1972, Contell & Kerr 1976, Peters et al. 1999, Paxton et al. 2001), there is a great amount of variation in whether the workers or the queen produces the males (Engels & Imperatriz Fonseca 1990, Inoue et al. 1999, Moo Vale et al 2001, Chapter 3). Because there does not seem to be variation in the genetic structure of stinglees bee colonies that would explain variation in male production by workers among species other factors must account for this variation. Besides the relatedness-based prediction for who should produce the males, predictions can be made based
on who can best exercise control of male production and also on colony level cost/benefit analyses. Moreover, current male production patterns in stingless bees may not reflect ongoing conflict, but could be a result of conflict of bygone times (Table 1).

As it is in the queen's interest to produce males, she may be more likely to do so when she is more capable to forcefully prevent the workers from reproducing. This might be the case when she is larger than the workers. Her larger size need not be fixed at emergence but could also be gained through physogastry, a largely swollen abdomen containing many eggs (Engels and Imperatriz Fonseca 1990). Of course the workers will be selected to resist her force. The bigger the difference between worker and queen size the better the queen should be able to manipulate workers.

Variation in the nature of the oviposition process could also affect whether the queen can prevent workers from producing males. In some species cells are prepared for oviposition one at a time (POP, Sakagami 1982). In other species the workers finish a whole row of cells at once (IOP). When many cells are provisioned at once the queen may lose control of who lays eggs in them (Zucchi 1993). Also, when more cells have to be oviposited at the same time, more workers are involved in the oviposition process and thus the queen would need to suppress more workers from
<table>
<thead>
<tr>
<th>Worker reproduction if:</th>
<th>Why</th>
<th>Kind of reason</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Colonies have a single once-mated queen</td>
<td>Worker relatedness is higher to nephews</td>
<td>Relatedness</td>
</tr>
<tr>
<td></td>
<td>than to brothers</td>
<td></td>
</tr>
<tr>
<td>2) Little queen/worker dimorphism</td>
<td>Queens cannot force workers behaviorally</td>
<td>Control</td>
</tr>
<tr>
<td>3) Many cells prepared for eggs at once</td>
<td>Queens fail to control oviposition situation</td>
<td>Control</td>
</tr>
<tr>
<td>4) Arms race won by workers</td>
<td>Workers escape queen control temporarily</td>
<td>Control</td>
</tr>
<tr>
<td>5) Large colonies</td>
<td>Many workers make worker reproduction</td>
<td>Control; Cost/benefit</td>
</tr>
<tr>
<td></td>
<td>less costly</td>
<td></td>
</tr>
<tr>
<td>6) Unproductive, inexperienced queen</td>
<td>Queen cannot force workers behaviorally,</td>
<td>Control; Cost/Benefit</td>
</tr>
<tr>
<td></td>
<td>best to focus her little reproduction on females</td>
<td></td>
</tr>
<tr>
<td>7) Seasonal male production</td>
<td>Workers can tell when to produce males</td>
<td>Cost/benefit</td>
</tr>
<tr>
<td></td>
<td>without hurting worker production</td>
<td></td>
</tr>
<tr>
<td>8) Abundant food</td>
<td>Worker reproduction will not greatly reduce colony productivity</td>
<td>Cost/benefit</td>
</tr>
<tr>
<td>9) Many excess queens</td>
<td>Replacing female eggs has less cost when they often become excess queens killed shortly after emergence</td>
<td>Cost/benefit</td>
</tr>
<tr>
<td>10) Other species in genus have it too</td>
<td>Pattern in worker reproduction determined in the past</td>
<td>Phylogeny</td>
</tr>
</tbody>
</table>
reproduction. As a consequence, with this sort of oviposition process there would be more worker production of males (Table 1).

If workers evolve to escape queen control and the queen in turn evolves new ways to suppress the workers this can lead to an arm race between the castes. Queen manipulation can happen through aggressive behavior backed by increased mass, or through pheromones (Ratnieks and Reeve 1992). Although pheromonal manipulation of workers by the queen should not be evolutionarily stable since workers should be selected to ignore such signals (Keller and Nonacs 1982), it could function if changing the chemical composition of pheromones is not too costly, allowing the queens to stay a step ahead of the workers (Foster et al. 2000). Queens could also develop skills to disturb workers closing cells in their oviposition and workers could avoid queen control by laying their eggs when the queen is not present. In this sort of arms race we expect variation in who wins across species. That is, some species should have queens successful in suppressing workers, and others should have workers succeeding ignoring queens. Moreover there could even be variation among closely related species (Crespi 1992). If variation in worker reproduction occurs for this reason, it may not be correlated with any easily measured colony or individual parameters.
Sometimes, control issues and cost/benefit issues make the same prediction. Larger colonies with more workers are predicted to have a higher level of worker reproduction. There are two reasons to expect this. First the cost of worker male production will be less to the colony and second because the queen is likely to be less able to control a larger number of workers (Table 1). The first half of this prediction depends on a decreasing benefit with number of workers and on a cost to worker reproduction in terms of reduced foraging efforts. It is a prediction that should hold both within and between species. The first prediction based both on control and on cost/benefit issues is that colonies where the queen does not function in an indefectible way will be more likely to have worker male production. The efforts of such queens should be focused on female production which only they can do, and they might not have the force to easily control workers (Table 1.)

There are three predictions exclusively based on costs and benefits that will favor worker production. First, if male production is highly seasonal, then workers should produce males during the season that lowers the risk of reducing the number of workers that would be produced by replacing the queen's female eggs with their own male eggs. This would be true if workers cannot distinguish between a male and a female egg of the
queen (Nonacs & Carlin 1990). So a more seasonal male production will favor worker male production. Second, when food is abundant, worker male production will not have as large of a cost to the productivity of the colony as it would if food were scarce. This is because when food is abundant the cost of reduced worker force is ameliorated relative to when food is scarce and more workers are needed. Of course the above mentioned predictions are not mutually exclusive. Seasonal male production can occur because at certain times of the year more flowers are available and more food is collected. Third, in some species many excess queens, up to 25% of all females, are produced which are then executed by the workers (Engels & Imperetriz Fonseca 1999). This is thought to be the result of female brood choosing to become queens rather than workers, a possibility open to females of Melipona where cells of developing workers and queens are identical (Ratnieks 2001). We expect substantial worker reproduction in such species since reproductive workers are more likely to be replacing a redundant queen, not a worker.

Finally, worker male production may be variable in the tribe, but at fixed levels in genera. This could have happened if the conflict over male production were settled evolutionarily a long time ago and a subsequent suite of characters followed that make worker reproduction unlikely or
impossible. In this case we would expect that phylogenetic relationships best
determine who produces the males, with closely related species sharing this
trait. Unfortunately, despite several attempts no single clearly resolved
phylogenetic tree is available for stingless bee species at this moment (Wille

To address the different predictions on the pattern of worker
reproduction we collected data published so far on male production known
from the literature on stingless bees.

Methods

To evaluate male production in stingless bees we collected data from
the literature. We gathered information on worker ovarian development, the
amount of male production, and the type of oviposition in the species. Most
published studies failed to include colony size (except for Trigona nigra
pauperea and Leurotrigona muelleri), and queen/worker body size ratios.
Therefore, for colony size, most data came from our personal observations
and personal communications on the particular species. Our estimation on
colony size was counting 50 bees in the colony in one area, then by going to
different parts of the nest we moved mentally the image of 50 bees and laid it over other areas in the nest were bees were moving around. We guessed the number of bees that were covered by colony structures, like involucrum or brood combs depending on how big the covering surface was. We calculated the ratio of queen-worker sizes by averaging the length from the tip of the head to the tip of the abdomen of three physogastric queens and three workers per species using enlarged pictures. For some species no pictures were available. In all those species queens are produced in bigger cells than workers and thus the physogastric queen had to be at least 1.5 to 1.8 times the size of a worker. For species with unknown queen/worker body size we used ratio 1.65. When no estimation of the proportion of worker reproduction was reported, according to whether investigators of behavioral studies mentioned we labeled them as substantial or small if the investigator reported it to be quite common or occurring with a low frequency respectively. We categorized species into three groups depending on their oviposition process. Some species build and oviposit cells mainly one at the time (provisioning and oviposition process, POP). Other species provision and oviposit their cells in groups (integrated oviposition process, IOP) and other species oviposit cells singly or in groups (facultative oviposition process, FAC). For analyses we treated FAC species and IOP species
together because FAC species usually have more than one cell ready for oviposition.

We did the analysis with average colony sizes and average worker reproduction previously reported in genetic studies. Because there is no accurate phylogenetic tree available to control for the most basic level of shared characteristics, we averaged characters within genera, making only one entry per genus in the analysis.

**Results**

We found published studies of male production from 22 species (Table 2). Twenty-one of those species are Neotropical and one is Australian. Twelve of these species had cells prepared one at a time for oviposition (POP), and 14 had a group of cells prepared together for oviposition (IOP). In two species workers could build one to a few cells at the same time (FAC). Colony size varied from 300 to 10,000 individuals per species. Functional physogastric queens varied from almost as small as workers to more than three times the size of a worker.
Table 2 shows that there is a great amount of variation in whether the workers or the queen produces the males. The lack of worker reproduction has been confirmed for a few species. In these species workers never have developed ovaries (*Frieseomelitta varia*) or workers do not develop their ovaries in the presence of a functional queen (*Trigona nigra pauparea, T. minagkabau, Neurotrigona mulleri*). Yet, even in species where workers have developed ovaries when a queen is present in a nest, workers do not always produce males (*Melipona beecheii, Schwarziana quadripunctata, Tetragonisca angustula*).

The differences in queen and worker body size did not correlate with the degree of worker reproduction (Spearman, p=0.60, N=13 genera). Worker oviposition was not significantly related to the number of cells prepared at a time (POP vs IOP) for oviposition (Mann-Whitney Test, p=0.39, N=10 genera). Worker reproduction did not correlate with colony size, measured as numbers of workers (Spearman, p=0.29, N=11 genera). *Melipona*, the genus with excess queens, on average had high levels of worker male production, although *Melipona beecheii* and maybe *Melipona fasciata* (just one colony studied for a short period) seem to be exceptions to this (Table 1). This partially supports the prediction that colonies where
Table 2: Stingless bee species for which it is known from the literature whether workers reproduce or not. The different columns indicate worker ovary development, the % of worker produced males (Sm = a small part, Sub = a substantial part), the type of study (B = behavioral, G = genetic), range of colony size, average colony size, the way of oviposition (POP = one cell is built and oviposited at the same time, IOP = few to many cells are oviposited at the same time, FAC = one or several cells are oviposited at the same time) and the source cited.

<table>
<thead>
<tr>
<th>Species</th>
<th>W ovaries</th>
<th>Avg % W males</th>
<th>Range W males</th>
<th>Type study</th>
<th>Colony size</th>
<th>Avg col sz</th>
<th># col studied</th>
<th>O/W sz</th>
<th>Ovip type</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Melipona beecheii</td>
<td>+ 0</td>
<td>G</td>
<td>300-3000</td>
<td>1650</td>
<td>13</td>
<td>1.2</td>
<td>POP Paxton et al. 2001</td>
<td></td>
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<tr>
<td>M. fasciata</td>
<td>+ 1</td>
<td>B</td>
<td>500-3000</td>
<td>1750</td>
<td>?</td>
<td>1.3</td>
<td>POP Inoue et al. 1999</td>
<td></td>
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<td>B</td>
<td>300-2000</td>
<td>1150</td>
<td>4</td>
<td>1.5</td>
<td>POP Sommeijer et al. 1999</td>
<td></td>
<td></td>
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<tr>
<td>M. quadrifasciata</td>
<td>+ Sub</td>
<td>B</td>
<td>500-3000</td>
<td>1750</td>
<td>1?</td>
<td>1.2</td>
<td>POP Tambasco 1971</td>
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<td>M. quadrifasciata</td>
<td>+ 64</td>
<td>55-70 G</td>
<td>300-2000</td>
<td>1150</td>
<td>2</td>
<td>1.2</td>
<td>POP Chapter 3</td>
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<td>M. rufiventris</td>
<td>+ Sub</td>
<td>B</td>
<td>500-3000</td>
<td>1750</td>
<td>?</td>
<td>1.2</td>
<td>POP Tambasco 1971</td>
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<td>M. subnitida</td>
<td>+ 39</td>
<td>0-100 G</td>
<td>500-3000</td>
<td>1750</td>
<td>14</td>
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<td>500-3000</td>
<td>1750</td>
<td>4</td>
<td>1.2</td>
<td>POP Koedam et al 1999</td>
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<td>M. scutellaris</td>
<td>+ 49</td>
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<td>1000-2000</td>
<td>1500</td>
<td>5</td>
<td>1.3</td>
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<td></td>
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<td>M. marginata</td>
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<td>15-85 G</td>
<td>500-1500</td>
<td>1000</td>
<td>3</td>
<td>1.5</td>
<td>POP Chapter 3c</td>
<td></td>
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<td></td>
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<td>Frisella Schrottkyi</td>
<td>+ 100</td>
<td>B</td>
<td>300-2500</td>
<td>1150</td>
<td>1</td>
<td>1.8</td>
<td>POP Fonseca &amp; Matos 1982</td>
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<td>Friseomelitta varia</td>
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<td>B</td>
<td>800-1600</td>
<td>1200</td>
<td>?</td>
<td>1.3</td>
<td>IOP Zucchi 1993</td>
<td></td>
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<td>Plebeia doryana</td>
<td>+ 16</td>
<td>0-57 G</td>
<td>800-5000</td>
<td>2900</td>
<td>14</td>
<td>1.9</td>
<td>IOP Machado et al. 1984</td>
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<tr>
<td>P. doryana</td>
<td>+ 0</td>
<td>G</td>
<td>800-5000</td>
<td>idem</td>
<td>1</td>
<td>1.9</td>
<td>IOP Chapter 3c</td>
<td></td>
<td></td>
<td></td>
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<td>P. remota</td>
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<td>B</td>
<td>800-5000</td>
<td>2900</td>
<td>6</td>
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<td>800-5000</td>
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<td>5</td>
<td>2.2</td>
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<td>P. saiqui</td>
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<td>1000-2000</td>
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<td>1.8</td>
<td>IOP Chapter 3c</td>
<td></td>
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<td>Paratrigona subnuda</td>
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<td>28-80 G, B</td>
<td>2500-5000</td>
<td>3750</td>
<td>6</td>
<td>1.7</td>
<td>IOP Chapter 5</td>
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<td>Scaptotrigona postica</td>
<td>+ 42</td>
<td>0-100 G</td>
<td>3500-8000</td>
<td>5750</td>
<td>3</td>
<td>1.5</td>
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<td>S. postica</td>
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<td>idem</td>
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<td>1.5</td>
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<td>Species</td>
<td>Q</td>
<td>G</td>
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<td>10000</td>
<td>20000</td>
<td>50000</td>
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<tr>
<td>S. postica</td>
<td>+</td>
<td>13</td>
<td>G</td>
<td>idem</td>
<td>idem</td>
<td>8</td>
<td>1.5</td>
<td>IOP</td>
<td>Paxton et al. 2001</td>
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<tr>
<td>S. barrocoloredensis</td>
<td>+</td>
<td>≤25</td>
<td>B</td>
<td>3000-7000</td>
<td>5000</td>
<td>?</td>
<td>1.6</td>
<td>IOP</td>
<td>Inoue et al. 1999</td>
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<td>0</td>
<td>G, B</td>
<td>500-2500</td>
<td>1500</td>
<td>16</td>
<td>1.5</td>
<td>POP</td>
<td>Chapter 4</td>
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<td>B</td>
<td>2500-7000</td>
<td>4750</td>
<td>?</td>
<td>+</td>
<td>FAC</td>
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<td>66</td>
<td>0-100</td>
<td>G</td>
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<td>5</td>
<td>+</td>
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<td>B</td>
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<td>5000</td>
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<td>3.3</td>
<td>IOP</td>
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<td>B</td>
<td>500-1000</td>
<td>750</td>
<td>1</td>
<td>+</td>
<td>IOP</td>
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<td>B</td>
<td></td>
<td></td>
<td>1</td>
<td>+</td>
<td>POP</td>
<td>Suka &amp; Inoue 1993</td>
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<tr>
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<td>0</td>
<td>B</td>
<td>500-1000</td>
<td>750</td>
<td>1</td>
<td>+</td>
<td>POP</td>
<td>Sakagmi &amp; Zucchi 1974</td>
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<td>Duckeola ghilianini</td>
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<td></td>
<td></td>
<td></td>
<td>1</td>
<td>+</td>
<td>POP</td>
<td>Sakagmi &amp; Zucchi 1968</td>
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<tr>
<td>Austroplebeia australis</td>
<td>+</td>
<td>Sm</td>
<td>G, B</td>
<td>3000-8000</td>
<td>5500</td>
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<td>+</td>
<td>POP</td>
<td>Drumond et al. 2000</td>
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worker-produced male eggs would be replacing excess queens instead of workers, will have more worker reproduction.

Studies that concentrated on worker male-production usually do not mention the status of their queens and whether male production occurred seasonally and when abundant food was available. Despite this other studies on for example Melipona beecheii, Scaptotrigona postica, Plebeia remota, P. droryana support the validity of these predictions (Beig 1972, Cortopassi-Laurino 1979, Bego 1990, Grosso et al. 2000, Moo-Vale et al. 2001). Colonies tend to produce males at times when there is plenty of food in the nest Thus male production is often seasonal depending on food availability (Beig 1972, Sakagami 1982, van Veen et al 1990, Grosso et al. 2000). Also, queens that poorly time their egg laying activities might lose male production to workers. Sometimes older queens do not react rapidly enough when a cell is ready to be oviposited as Sommeijer & van Buren showed it for Melipona rufiventris (1992), and recently mated young queens need to ‘learn’ how to interact with workers and when to lay their eggs (Beig 1972).

The number of males produced by workers varies across species. Moreover, according to all 11 genetic studies that showed that worker reproduction in stingless bees, the amount of worker reproduction varies between colonies of the same species as well (Table 2). In some colonies
workers might not reproduce at all, while in other colonies they can produce 100% of the males.

Worker reproduction levels tended to be similar within genera. *Melipona* workers, except for *Melipona beecheii* and *M. fasciata*, had high worker reproduction on average (Table 1). Similarly, in both *Scaptotrigona* species workers substantially contributed to males. By contrast, *Plebeia* species generally had low rates of worker male production.

**Discussion**

Male production by workers is widespread in stingless bees, but not universal. Of the 13 species with workers possessing ovarian development for which genetic data are available, only *Melipona beecheii*, *Plebeia saiqui*, and *Schwarziana quadripunctata* had no male production by workers. Behavioral studies cannot predict with certainty the amount of worker produced males. Yet studies that included several colonies for each species and where investigators opened cells are probably accurate about stating whether workers reproduce. Thus it is likely, there is also no worker
reproduction in *Tetragonisca angustula* (Grosso et al. 2000). This list might be extended with more genetic studies.

Not only is there variation of worker reproduction between species, but also within species. All genetic studies that found worker reproduction reported substantial levels of within species variation (Table 2). How well can variation in worker male production within species be explained by the factors in Table 1? To fully address this we will generally need larger sample sizes and more information on the colonies than are presently available for this fascinating but understudied tribe.

The lowest queen/worker dimorphism is found in *Melipona*, where queens are actually smaller than workers until the queens become physogastric. Even with physogastry, *Melipona* queens are smaller on average relative to their workers than is the case for other species (Table 2). This is the only genus that produces queens from the same size cells as workers and also the only one with a great excess of queens. We hypothesize that the latter will reduce the cost of worker male-production. Perhaps both the decreased cost of worker male-production and the decreased ability of queens to manipulate workers, allows for high levels of worker reproduction in this genus. Across genera, however, there was no support for the
hypothesis that increased queen size relative to workers decreased worker production of males.

The hypothesis that queens would be less able to maintain control of worker reproduction when many cells are prepared for oviposition simultaneously (IOP species) was not supported. *Melipona* with high worker oviposition has POP and *Plebeia* with low worker oviposition has IOP. Thus both are counter to the expectations of the hypothesis. There was no consistent overall pattern including all genera either (Table 2).

In colonies with many workers, the cost of worker male production should be less consistent with predictions, species with more workers should be more likely to have worker male production. However our comparisons across genera do not support this. The importance of the correlation between worker number and male production seems to be supported within species though. Previous work indicates that colonies with a big worker force seem to produce more males than small colonies (Cortopassi-Laurino 1979, Camillo-Attique 1977, Machado et al. 1984, Sommeijer et al 1984b, van Veen et al. 2001, Grosso et al. 2000, Moo-Vale et al. 2001).

Though, not much focus was paid to functionality of the queen, in *Melipona rufiventris* and *Scaprotrigona postica* it was confirmed that worker reproduction occurs when the queen is not timing when the workers
are ready with cell provisioning (Camilo-Atique 1977, Sommeijer et al. 1984b, Sommeijer & van Buren 1992). Many studies mention that workers would produce males in the season of maximal male production. This is when workers are least likely to be replacing female destined eggs with male destined eggs. Yet strong colonies of *Melipona beecheii, Scaptotrigona postica, Plebeia remota, P. droryana* sometimes produce males during the whole year and thus male production might indirectly depend on the amount of resources in the colonies (Beig 1972, da Silva 1977, van Veen et al. 2001). Some studies suggest that males are produced when food is abundant. As consequence of this workers thus might reproduce in the same period (Beig 1972, Koedam et al 1999, Chapter 5).

Although we lack an accurate phylogenetic tree for stingless bees, phylogenetic history appears to have an impact on worker male production. For example, species within the genera *Melipona, Scaptotrigona* and *Plebeia* show similar degrees of worker reproduction. This could be because related species might have similar ecology resulting in similar degree of worker reproduction. Phylogenetic relationships do not seem to have a strong effect as the range of worker reproduction sometimes differs strongly between colonies of the same species. The maximum proportion of worker produced
males may be phylogenetically resolved, while costs and benefits could be responsible for how much the workers actually invest in reproduction.

The extensive occurrence of worker male production in stingless bees is exactly what we predict if the genetic interests of workers control male production. Across most genera male production by workers is reported and sometimes it is predominant. This is in marked contrast to the situation for honeybees. We were less successful in determining what predicted variation in male production. In some species workers refrain from reproduction because of the costs involved. Worker reproduction is also phylogenetically constrained. It will be very interesting to see if additional genetic data will allow us to account for variation in worker male production.
CHAPTER VIII

General conclusions

From the previous chapters I conclude that although most stingless bees have one singly mated queen, workers contribute to male production equally in different colonies and species. In this thesis I attempted to find an answer to what causes workers to refrain totally or partially from reproduction. For this, I looked and analyzed male production and behavioral interactions on different levels; colony, species and across species.

Chapter 3 discusses male production by workers in eight stingless bee species. In some stingless bees workers reproduce in others they do not. To assess male production by workers the likelihood method was more accurate than the exclusion method.

In Chapter 4 I analyzed data on the colony and species level in Schwarziana quadripunctata. The results did not confirm the predictions that the behavioral interactions between workers and queens in S. quadripunctata colonies during the male producing period is more aggressive than the behavior outside the male producing period. Although
the results can be explained in various ways the lack of elevated aggression could most probably due to the fact that workers do not reproduce in this species

In Chapter 5 I investigated worker reproduction and behavioral interactions in Paratrigona subnuda colonies. In P. subnuda workers substantially contributed to male production, although they did not produce all of the males. The behavioral analysis showed that workers reproduced not during, but after the oviposition process. Even though the queen was aware of worker reproduction in many cases there was no escalation of aggression between reproductive workers and the queen. The queen tried to push reproductive workers aside. Workers on their turn just gave way to the queen in a part of the incidents, after queen departure though all workers finished laying eggs successfully. Although the queen does not have control over worker reproduction in P. subnuda workers do not dominate male production completely, most probably because of the costs involved.

Since I did not find a sign of elevated aggression between the workers and the queen in S. quadripunctata and P. subnuda I wondered whether it was just the property of the formal species or that it is valid for stingless bees in general. In Chapter 6 thus I studied behaviors and reproduction among species. We compared queen-worker interactions and the amount of
worker reproduction in eleven bee species. The results do not confirm the
predictions that in species where both the queen and the workers produce
males, interaction between workers and queens are more aggressive than in
species where only the queen produces the males. Maybe because laying
workers might usually not be involved in the oviposition process, elevated
aggression does not make sense. Behaviors that seem aggressive to us have
other, non-aggressive communicative functions to the bees.

In Chapter 7 I synthesized my results with all results known so far
from the literature concerning worker reproduction. I also considered
ecological factors like colony size, way of oviposition, and queen’s size
versus workers in the comparison. The role of phylogenetic relationship
between species was considered as well. The results across genera indicate
that the amount of worker reproduction is not related to queen size nor the
way of oviposition, and also not to colony size. The costs involved in worker
reproduction could explain why workers refrain from reproduction in some
species and not in other. To confirm that costs are important the role of other
ecological factors on worker reproduction have to be studied and
understood. The amount of worker reproduction in stingless bees might also
be phylogenetically determined. Species belonging into the same genus
show similar level of worker reproduction. Species within the same genus
might have similar colony ‘life style’ with similar costs to worker reproduction.

Although this thesis added a lot of information about worker reproduction to our knowledge on stingless bees, clearly more studies are needed to find solutions to conflict related question. On the same species level the behavioral interactions of species that have worker reproduction need to be studied in periods with no male and periods with male production. Expectations of these studies need to be adjusted, most probably because the costs involved bees will not show overt aggression, but conflict will manifest itself in rather subtle ways. Demographical factors, such as queen’s age and productivity. Furthermore, the role of communication with regards of worker-queen interactions needs to be studied in great detail. Ultimately, a good phylogenetic tree with high resolution is needed for comparative studies.
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