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

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Abstract

The genetic structure of social insect colonies is predicted to affect the balance between cooperation and conflict. Stingless bees are of special interest in this respect because they are singly mated relatives of the multiply mated honeybees. Multiple mating is predicted to lead to workers policing each others' male production with the result that virtually all males are produced by the queen, and this prediction is borne out in honey bees. Single mating by the queen, as in stingless bees, causes workers to be more related to each others' sons than to the queen's sons, so they should not police each other. We used microsatellite markers to confirm single mating in eight species of stingless bees and then tested the prediction that workers would produce males. Using a likelihood method, we found some worker male production in six of the eight species, although queens produced some males in all of them. Thus the predicted contrast with honeybees is observed, but not perfectly, perhaps because workers either lack complete control or because of costs of conflict. The data are consistent with the view that there is ongoing conflict over male production. Our method of estimating worker male production appears to be more accurate than exclusion, which sometimes underestimates the proportion of males that are worker produced.

Keywords: likelihood method, male parentage, Meliponinae, microsatellites

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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 explains not only cooperation among relatives but also how selfish behaviour leading to conflicts can take place (Hamilton 1964; Hamilton 1972).

Conflicts in insect societies often arise because females are not related uniformly 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 related equally 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 (Woyciechowski & Lomnicki 1987; Ratnieks 1988; Ratnieks & Visscher 1989). In contrast, in species where the queen mates just once, such as stingless bees (da Silva *et al*. 1972; Contel & Kerr 1976; Peters *et al*. 1999; Palmer *et al*. in press) 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 & Strassmann 1998).

Previous behavioural 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*.

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1984a; van Benthem *et al*. 1995; Inoue *et al*. 1999; Koedam *et al*. 1999; Grosso *et al*. 2000) even though, according to genetic studies, the single, once-mated queen rule appears to be general across the tribe (Peters *et al*. 1999; Palmer *et al*. in press). 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 enough 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 (Sakagami *et al*. 1963; Sakagami & Zucchi 1974; Suka & Inoue 1993). However, some behavioural observations suggest that, even in species where workers have developed ovaries, they might not always lay male eggs (Sommeijer *et al*. 1984b; Sakagami & Zucchi 1974; Suka & Inoue 1993; Inoue *et al*. 1999; Grosso *et al*. 2000). However, behavioural 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 (Tóth *et al*. submitted, b). 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 behavioural data (Contel & Kerr 1976; Machado *et al*. 1984; Drumond *et al*. 2000; Paxton *et al*. 2001; Palmer *et al*. in press; Tóth *et al*. submitted, a, b). The results here are mixed; worker production of males occurs in some species, but in others the queen seems to produce all the males, but even here the results can be ambiguous. Most studies use exclusion methods, but these are one-sided. A queen can be excluded as mother of a worker-produced male because the worker can transmit genes to her son that the queen does not possess. However, workers cannot be excluded as possible mothers of queen-produced males because any allele transmitted by the queen to her son will also have been transmitted to workers. Thus, the only way to estimate worker production by this method is to count those who are excluded as queen-produced. This is acceptable if the exclusion power of the molecular markers is high. However, if exclusion power is lower, the method is biased; worker males may be assigned falsely to the queen but not *vice versa*.

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 vs. queen production. The primary goal of our study was to investigate parentage of male brood in a number of species to obtain 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 (9–20 per colony) and 412 males (from five 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 polyacrymalide gels following the protocols of Strassmann *et al*. (1996). To minimize error, gels were scored twice independently (by two people). The results were compared and discrepancies were reconciled, a process that sometimes included reconducting 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 & 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 (CI) 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 (Tóth *et al*. submitted, a) which is a modified version of that used by Arévalo *et al*. (1998), designed originally for multiple-queen species. For singly mated single-queen 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).

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

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

The likelihood, L, for any value hypothetical value of Q is calculated as $L = K \prod_{males}\left(Q\prod_{loci} f_{qi} + (1-Q)\prod_{loci} f_{wi}\right)$. K is a multinomial constant that never has to be calculated because it multiplies all Ls by the same value, and cancels out 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 × B mating has likelihood p^2q and a BB × 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 assume one mating for some offspring and the other mating for the rest. An Excel spreadsheet to apply these calculations is available from DCQ.

It is worth noting that this method should never be worse than exclusion, because exclusion information is fully incorporated: cases where $L = 0$. Exclusion methods effectively discard additional information from cases where $L \neq 0$.

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 (da Silva *et al.* 1972; Peters *et al.* 1999; Strassmann 2001; Palmer *et al.* 2002). 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 were very frequent in the population.

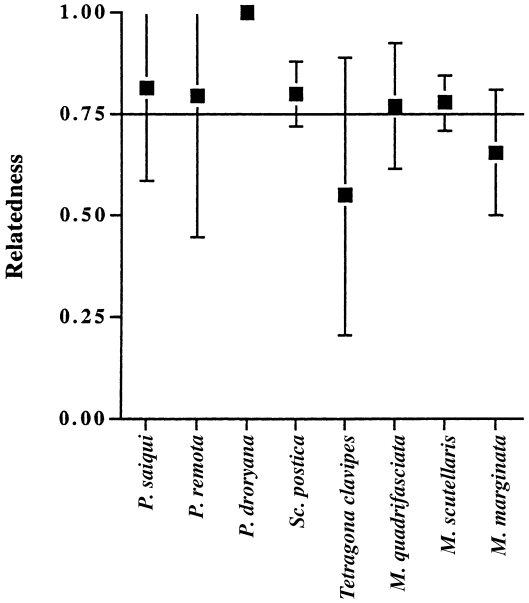

Fig. 1 Relatedness estimates (± 95% CI) for workers of eight stingless bee species.

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

Species	Colony	Males excluded	Males genotyped	Exclusion Q estimate	Likelihood Q estimate
P. remota	colony 3	1	10	0.90	0.80
S. postica	colony 1	1	15	0.93	0.74
	colony 2	7	18	0.61	0.58
M. quadrifaciata	colony 1	0	22	1.00	0.40
	colony 4	0	24	1.00	0.32
M. scutellaris	Colony 1	2	6	0.67	0.56
	colony 5	11	13	0.15	0.00
M. marginata	colony 1	2	11	0.82	0.76
	colony 2	4	16	0.75	0.64
	colony 3	8	18	0.56	0.40
T. clavipes	colony 1	0	10	1.00	0.00
	colony 2	0	9	1.00	0.18
	colony 3	0	7	1.00	0.00
	colony 5	0	11	1.00	0.44

Male production

Workers produced some of the males in six of the eight species (Fig. 2). In a few colonies, workers apparently produced all the males (maximum likelihood $Q = 0$), but in no species was this true overall. Variation among colonies was common. Both *M. scutellaris* and *T. clavipes* appeared to include at least one colony with complete worker production, at 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 (five) 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 *T. clavipes* curves are somewhat 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 implausible.

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

Attributing males to the queen when they are not 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. Note that, when they differ, the likelihood estimates of queen reproduction are always lower than the exclusion estimates. This is because the likelihood procedure includes queen exclusion (cases of zero likelihood) but is also able to infer some worker reproduction among nonexcluded males. Presumably, with increasingly good genetic information, all nonexcluded 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 *T. 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 × A and the other two were AB × 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 previous evidence suggests that workers produce males in this group (Beig 1972; Sommeijer *et al.* 1984b; Imperatriz-Fonseca & Matos de Peixoto Kleinert 1996; Inoue *et al.* 1999; Koedam *et al.* 1999), but there have been few genetic studies (Contel & Kerr 1976; Machado *et al.* 1984; Drumond *et al.* 2000; Paxton *et al.* 2001; Palmer *et al.* 2002; Tóth *et al.* submitted, a, b) to confirm this. These genetic studies suggest that workers sometimes, but not always, produce males.

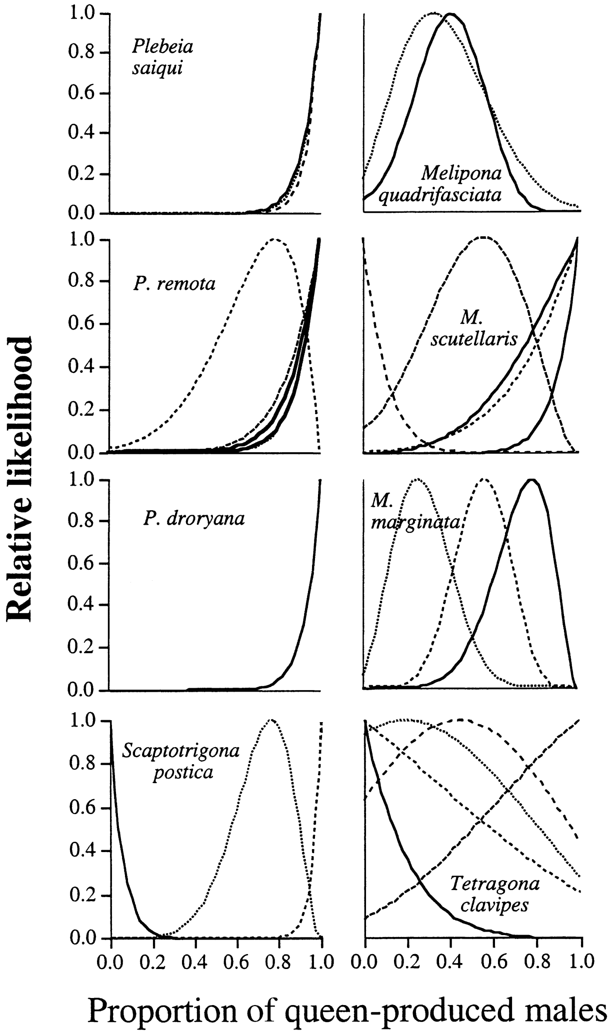

Fig. 2 Curves of relative likelihoods for the fraction of queen-produced males for colonies of eight stingless bee species. Each curve represents one colony, except for the two heavy-line curves of *P. remota*, each of which represents two curves.

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, although 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 to choose between mainly queen or mainly worker production of males. The approach we use in this paper is closer in spirit to that used in earlier Brazilian studies (Contel & 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 corrected for the bias by scaling 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 × B vs. BB × 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 queens share reproduction within colonies of *T. clavipes* is not solid, while the same assertion can be made with confidence for *M. marginata*.

Our results show that workers produce some of the males in six of the eight species studied. There is clearly an average difference, in the predicted direction, from honey bees' 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 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), although *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*, *Sc. postica* and for *Paratrigona subnuda* (Contel & Kerr 1976; Machado *et al.* 1984; Paxton *et al.* 2001; Tóth *et al.* submitted, b). 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.

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This work is part of a broader project on conflicts over male production in stingless bees, the PhD project of Eva Tóth. The Houston laboratory works predominantly on the evolutionary outcome of conflicts in organism that cooperate. The São Paulo laboratory works on the ecology of stingless bees.