Worker reproduction in the ant *Formica fusca*

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Abstract

We studied the kin conflict over male parentage in the ant *Formica fusca*. The conflict arises because each worker and queen is most related to her own sons and is thus predicted to lay eggs. Microsatellite analysis of eggs revealed that workers laid eggs in more than half the queenright experimental nests. Nevertheless, almost exclusively diploid offspring were reared in the presence of a queen. This also occurred when worker-laid haploid male eggs were experimentally introduced in to the nests. Because our experimental setup allowed us to exclude the possibility of queen policing, we conclude that worker laid eggs are removed by other workers, either as a response to their parentage or gender. Our results suggest that worker reproduction in *F. fusca* is ultimately an interplay of conflicts over male parentage and sex allocation and that both worker and self policing have roles as proximate mechanisms of resolution.

Introduction

Kin selection theory (Hamilton, 1964) provides a theoretical framework for understanding the evolution of co-operation among related individuals. However, the genetic perspective adopted in inclusive fitness arguments also predicts within-group conflicts to arise whenever the co-operating individuals are not genetically identical (Hamilton, 1964; Trivers & Hare, 1976). This is because, all other things being equal, each individual gains most by favouring the offspring produced by their closest relatives (Hamilton, 1964; Trivers & Hare, 1976). In insect societies conflicts may arise over reproductive status, sex allocation, caste fate or brood composition (Trivers & Hare, 1976; Bourke & Franks, 1995; Crozier & Pamilo, 1996; Bourke & Ratnieks, 1999; Ratnieks, 2001; Wenseleers *et al.*, 2003) and may involve individuals of the same or different castes. In social hymenoptera workers of many species have fully functional ovaries, although they lack a sperm receptacle and therefore cannot mate. Such workers can lay unfertilized eggs, which, because of the haplodiploid sex determining mechanism of Hymenoptera, can develop into fully

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functional males. Therefore, conflicts over male parentage may arise either between queens and workers, or among workers.

The conflict over male parentage arises because a female (worker or queen) always values her own sons more than anybody else's [$r(sons) = 0.5$]. If workers were to produce males, queens would trade sons for less closely related grandsons ($r = 0.25$) and a conflict between queens and workers arises if the workers pursue their own interests. For workers the situation is more complex, as they choose between their own sons, nephews and brothers. In this case colony kin structure determines which parties eventually come into conflict. In a colony with one, singly mated queen, workers are full sisters ($r = 0.75$) and therefore are more closely related to their nephews [$r(wm) = 0.375$] than their brothers [$r(qm) = 0.25$]. As a result, the conflict over male parentage arises between workers and the queen, because workers value their nephews more than the queen's sons (Ratnieks, 1988; Pamilo, 1991). Conversely, if the colony contains multiple related queens (polygyny), or the queen is multiply mated (polyandry), the conflict arises between the individual worker and the worker collective in union with the queen. When the effective number of queens [Ne, which takes into account both the number and relatedness of queens and the variance among their reproductive shares (Queller, 1993)] in the colony exceeds a threshold value $1 + 1/r(q)$

where $r(q)$ is the relatedness among nest mate queens, the average relatedness of workers to worker-produced males will be lower than that between workers and queen-produced males (Pamilo, 1991; Bourke & Franks, 1995). In this case the optima for queens and worker collective converge and the majority of workers are predicted to favour queen-laid males and control the reproduction of their fellow workers ('worker policing' Ratnieks, 1988; Pamilo, 1991).

Worker policing may occur via egg removal or aggression directed towards fertile individuals (Ratnieks, 1988, 1993) and can lead to a stable state of workers laying eggs and other workers removing the eggs. This is the case for example in the honeybee *Apis mellifera* (Ratnieks & Visscher, 1989; Ratnieks, 1993; Visscher & Dukas, 1995; Visscher, 1996). Selfish reproduction is likely to entail costs in terms of reduced colony performance, but evidence remains elusive (but see Cole, 1986). Policing reduces the potential benefits of selfish acts. Over evolutionary time selection may eventually lead to complete sterility of workers as is the case in several highly polygyne ant species [e.g. in the genus *Solenopsis* (Bourke, 1988a)] and two species of monogynous stingless bees (Tóth *et al.*, 2004). Indeed, mutual policing mechanisms and self restraint (self policing) are an essential part in the evolution of co-operation of independent units towards more complex structures (Frank, 1995, 1996; Maynard-Smith & Szathmary, 1995; Reeve & Keller, 1997).

Worker reproduction and worker policing are well documented in bees and wasps, both in agreement and in contradiction with relatedness predictions. As predicted on relatedness grounds, worker policing occurs in the polyandrous honeybees *Apis mellifera, A. florea* and *A. cerana* (e.g. Ratnieks & Visscher, 1989; Ratnieks, 1993; Visscher, 1996; Halling *et al.*, 2001; Oldroyd *et al.*, 2001). In polyandrous *Vespula vulgaris* worker laid eggs are policed, but because of low effective paternities, workers are equally related to queen-produced and worker-produced males (Foster & Ratnieks, 2001). Conversely, workers produce males in *Dolichovespula* wasps and some stingless bees (*Paratrigona subnuda, Melipona quadrifasciata, M. scutellaris, M. marginata, Tetragona clavipes, Scaptotrigona postica* and *Plebeia remota*), where colonies are headed by one singly mated queen (Foster & Ratnieks, 2001; Foster *et al.*, 2001; Tóth *et al.*, 2002a, b). Finally, again in accord with relatedness predictions, in *Dolichovespula saxonica*, worker policing only occurs in the colonies headed by a doubly mated queen, whereas workers produce males in colonies headed by a singly mated queen (Foster & Ratnieks, 2000). In opposition to the predictions based on relatedness incentives all males are queen-produced in some monandrous stingless bees (*Plebeia saiqui, P. droryana, Trigona clypearis, T. hockingsi, T. mellipes,* Palmer *et al.*, 2002; Tóth *et al.*, 2002a) and the monandrous hornet *Vespa crabro*, in which worker policing occurs, probably due to colony level costs of worker reproduction (Foster *et al.*, 2000). Similarly,

worker policing occurs both as frequent worker-worker aggression and egg removal in some ant species, although colony kin structure predicts the opposite (review in Monnin & Ratnieks, 2001). Also in the wood ant genus *Formica*, worker reproduction seems to be absent in a number of species although male production by workers is predicted on genetic grounds (Walin *et al.*, 1998).

Several factors may lead to the absence of worker reproduction, although predicted based on relatedness incentives. Genetic relatedness only sets the potential for queen-worker or worker-worker conflict in societies (*potential conflict* sensu Ratnieks & Reeve, 1992), whereas the expression of conflicts (*actual conflict*) as well as their outcome is determined by asymmetrical constraints and benefits for the parties involved (Ratnieks & Reeve, 1992). Such asymmetries may occur in numbers, the quality of information and most importantly the reproductive potential (Hamilton, 1966; Ratnieks & Reeve, 1992; Sundström & Boomsma, 2001). A fertile queen is likely to be irreplaceable and thus more valuable to the colony than a single, somewhat redundant worker (Ratnieks & Reeve, 1992; Bourke & Franks, 1995). Also, as the only function of a queen is reproduction, queen fertility does not introduce colony level costs, whereas the number of reproducing workers may entail costs through decreased colony performance (Cole, 1986; Ratnieks, 1988; Ratnieks & Reeve, 1992; Bourke & Franks, 1995). In ants most genetic data on worker reproduction is based on samples of pupae (Heinze *et al.*, 1997; Evans, 1998; Walin *et al.*, 1998; Villesen & Boomsma, 2003), whereas little data are available on worker egg-laying (but see Hammond *et al.*, 2003). Hence, although no worker-produced males have been found among the mature brood the conflict may still be expressed by workers attempting to lay eggs.

Also kin conflicts other than those over worker reproduction may affect the production of worker-laid males. As recent verbal arguments (Walin *et al.*, 1998) and formal models (Foster & Ratnieks, 2000; Hammond *et al.*, 2003) suggest, male production by workers may be affected by the conflict over sex ratio. Sex ratio biasing by workers may select for worker policing and consequently for self policing, but this depends on both colony and population level patterns of sex allocation and worker relatedness towards females and males (for details see Foster & Ratnieks, 2000; Hammond *et al.*, 2003).

Our aim is to find out whether the conflict over male parentage is expressed through worker egg-laying in the ant *Formica fusca* (Linnaeus) and whether worker egg-laying and the fate of worker laid eggs are controlled by queen or workers. We combine genetic data on the parentage of male eggs and pupae with data from experimental manipulations to show that the potential conflict is expressed and the reproductive potential of the workers is realised as worker egg-laying in the colonies. Furthermore, our experimental results demonstrate that in the laboratory colonies rear almost exclusively diploid

offspring when the queen is present. Male eggs laid by workers are discriminated against either as a response to their parentage or a by-product of sex ratio conflict.

Materials and methods

The study species, *F. fusca*, has colony sizes reaching 1500 workers and usually one or a few queens (Collingwood, 1979; Savolainen, 1990; Czechowski *et al.*, 2002; Hannonen *et al.*, 2004). The workers are fertile and start laying eggs shortly after colony orphanage and rear these males into adults (H. Helanterä & L. Sundström, University of Helsinki, personal observations). Despite the apparent reproductive potential of workers and their genetic interest to produce sons, no worker-laid males have been found in queen right field colonies (Olsson, 1999, K. Trontti, L. Sundström, unpublished data).

Study colonies

Colonies of *F. fusca* were collected near Tvärminne zoological station in southern Finland in 1998–2000. The collection areas comprised a bog and a logged area (Populations Old and Young, respectively in Hannonen *et al.*, 2004). Given the observed average relatedness among queens of 0.57 ± 0.04 for Old and 0.34 ± 0.04 for Young (Hannonen *et al.*, 2004), the respective threshold values for preferential rearing of queen-laid males given by $Ne = 1 + 1/r(q)$ (Pamilo, 1991) are 2.75 and 3.94. The observed mean values of Ne 2.05 (2.01; 2.09) for Old and 4.44 (4.13; 4.66) for Young (means and 95% CI, Hannonen *et al.*, 2004) are, respectively, below and above the threshold value. This means that each worker should prefer her own sons, but the preferred source of males for the worker majority differs between the populations.

A total of 106 field colonies (86 from Young, 20 from Old) were excavated in late April to early May when queens gather near the surface before the onset of egg laying. The material comprising queens and workers was brought to the laboratory where 156 laboratory nests (34 from Old, 122 from Young) were established in plastic trays ($40 \times 30 \times 15$ cm) with peat, some of the original nest material, *Sphagnum* moss for maintaining humidity, and a ceramic plate (15×15 cm) as a nest site (below, 'nest' will be used to denote laboratory nests and 'colony' field colonies). Throughout the experiments nests were fed with standard Bhatkar–Whitcomb diet (Bhatkar & Whitcomb, 1970) and moistened daily with a spray can.

Experiment I: The parentage of eggs

1. To assess whether workers lay eggs in the presence of a queen we sampled eggs from 26 queen right (single queen) nests (from 17 field colonies) for microsatellite analysis. Once the first eggs appeared a sample of about 50 eggs was collected from each nest and stored in 94%

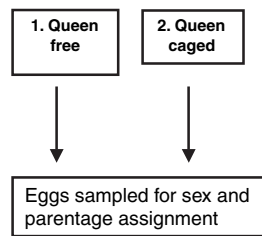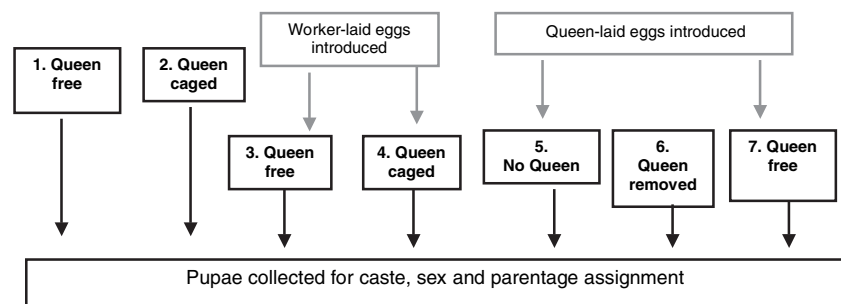ethanol. The presence of worker laid eggs indicates that the conflict is expressed.

2. Queen right laboratory nests were divided into two treatments to see if queens affect worker egg-laying chemically or physically or if the workers remove worker-laid eggs. In the control treatment ($n = 16$, from 11 field colonies) the queen was able to move freely and interact with workers and brood in the nest, whereas in the 'box' treatment ($n = 11$, from eight field colonies) the queen was confined in a small (c. $5 \times 10 \times 10$ cm) plastic box within the nest. The box walls were perforated with holes large enough to allow the passage of workers, but small enough to prevent the queen from escaping. Eggs were sometimes seen within the box, but they were always sampled from the outside. The absence of worker laid eggs in both treatments indicates either worker policing or chemically mediated queen effects through pheromonal suppression of worker reproduction or self restraint as a worker response to the presence of a queen (Keller & Nonacs, 1993). The presence of worker laid eggs in the box treatment but not in the control indicates physical queen policing.

Experiment II: Conflict resolution

To study the effects of different social conditions on the final brood composition, we set up a second experiment, consisting of queen right and orphaned nest fragments. These were subjected to seven treatments (1–7 in Fig. 1) designed to address the following questions:

1. To test whether any worker-produced brood develops into adulthood in unmanipulated conditions, nests similar to control (treatment 1, $n = 19$, from 18 field colonies) and Box treatments (treatment 2, $n = 25$, from 24 field colonies) above were set up and the brood was allowed to mature until pupation. As above, the absence of worker-laid pupae in both treatments would support either chemical queen effects or worker policing, whereas the presence of worker laid pupae in the box treatment but not in controls would support physical queen policing.

2. To test whether workers remove worker laid eggs in the presence of a queen, we transferred about 50 worker laid eggs from the orphaned nest fragments into two types of queen right nest fragments (always originating from the same field colony). In treatment 3 ($n = 19$, from 19 field colonies) the queens were free and in treatment 4 ($n = 15$, from 15 field colonies) the queens were caged as described above. Under queen control or worker control (mutual policing or self policing) we expect only queen-produced offspring to develop into pupae in both types of queen right nest fragments. Conversely, if queens physically interfere with worker-laid brood, we expect to find only queen-laid pupae in the queen right nest fragments with the queen at liberty, whereas at least some worker-laid males could be expected among the pupated brood in the queen right nest fragments with a caged queen.

Experiment I:

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┌──────────┐   ┌──────────┐
│ 1. Queen │   │ 2. Queen │
│   free   │   │  caged   │
└────┬─────┘   └────┬─────┘
     │              │
     ▼              ▼
┌──────────────────────────┐
│  Eggs sampled for sex and │
│  parentage assignment     │
└──────────────────────────┘
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Experiment II:

Fig. 1 Treatments, manipulations and collected samples.

3. To test whose offspring the workers raise when given a choice, we transferred eggs laid in queen right nest fragments (assuming that most of these are queen laid) into orphaned nest fragments, where the workers had already commenced egg laying on their own (treatment 5, $n = 23$, from 22 field colonies). To compare this with a situation where workers do not have their own brood present initially, we removed queens from queen right nests after the first 50–100 eggs had been laid (treatment 6, $n = 31$, from 30 field colonies).

4. Finally, to control for effects of these manipulations, we reciprocally transferred eggs between queen right nests originating from the same field colony (treatment 7, $n = 10$) to verify that transferred eggs were not destroyed as a response to the transfer treatment itself, but because of the parentage (or sex, see Discussion) of the egg.

The experiments were terminated and pupae biomass and caste and sex ratios were assessed when all offspring had pupated. All queens and pupae, as well as 12 workers from each original field colony, were stored at −20 °C for genotyping to estimate relatedness and assess parentage.

Sex and parentage assignment

The sex and parentage of eggs was assigned based on the genotypes of the queens and their male mates. The genotypes of the male mates of the queens were obtained from the DNA stored in the spermathecae, or if the spermathecae DNA did not amplify, inferred from diploid offspring genotypes (heterozygous eggs or worker pupae). Individuals were genotyped at six DNA-microsatellite loci (FL12, FL20, Chapuisat, 1996; FE13, FE17, FE19 and FE21, Gyllenstrand *et al.*, 2002). For extraction, PCR and gel procedures see Hannonen & Sundström (2002).

We first scored the queens, their spermathecae and 12 workers per colony at all six loci, to identify for each nest a diagnostic locus that allowed both discriminating between worker-laid and queen laid eggs, and determining the sex ratio of queen-produced offspring. Thus, to assess the proportion of worker-produced offspring we chose a locus where at least some workers carried an allele different from either of the two alleles carried by the queen. Offspring with a homozygous genotype carrying an allele different from the queen alleles were necessarily worker-laid males ('Worker-laid' in Fig. 2). As workers carried alleles similar to those of the queen, a proportion of worker-laid eggs cannot be distinguished from queen-laid male eggs. The proportion of such eggs was calculated as the product $\Pi\ p(i)$, where $p(i)$ is the proportion of queen alleles in workers for each loci genotyped in a colony. The corrected number of worker-laid eggs was obtained by dividing the observed number by $1 - \Pi p(i)$. The corrected numbers were used for all subsequent statistical analyses.
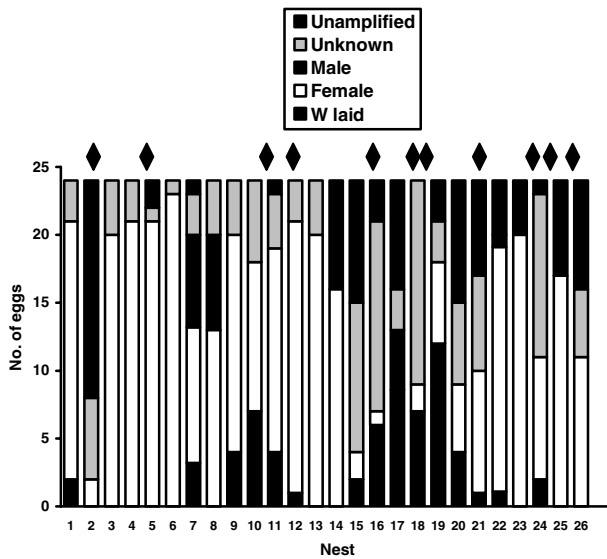
Fig. 2 Egg sex ratios and parentage in the experimental nests. Colonies 9–12 originated from a single field colony, as did 15–20. Colonies marked with a diamond were in the 'Box' treatment, the rest were controls.

To assess the sex of offspring we chose a locus where the male mate(s) of the queen carried allele(s) different from the queen. Offspring heterozygous at any locus were diploid and thus necessarily produced by the queen ('Female' in Fig. 2). When the queen had mated with male(s) carrying alleles different from herself, individuals with a homozygous genotype carrying an allele similar to those of the queen were assigned as a male ('Males' in Fig. 2). Whenever queens and workers shared alleles, the parentage of these males could not be determined. When the DNA in the eggs did not amplify at the diagnostic loci, individuals homozygous at the other loci with alleles similar to those of the queen were either males of unknown origin or homozygous females and consequently assigned as unknown. ('Unknown' in Fig. 2) The caste and sex of pupae were assessed based on morphology. Female sexual and worker pupae were certainly queen produced and male pupae were genotyped as described above to assign the parentage.

Statistical methods

When several laboratory nests originating from one field colony were used in the same treatment, we used the mean values for these nests in the analyses to avoid pseudoreplication. The results were qualitatively similar whether this was done or not.

We checked the normality of the variables using Wilk–Shapiro tests and used nonparametric tests for the variables which were not normally distributed (Wilk–Shapiro < 0.85) after ln-transformations. We analysed the numbers of worker-laid eggs in Experiment I and the ratio of all queen-produced vs. worker produced pupae in Experiment II using general linear models (GLM). This ratio was used instead of the actual numbers of worker-produced male pupae because the numbers were too low for formal analyses. This ratio was calculated as

$$M_q/M_w$$

where M_q is the biomass of queen-produced pupae and M_w the biomass of worker-produced pupae. Where no worker-produced pupae were found, we used M_q as the ratio to avoid having zero in the denominator. Males of unknown parentage (because of lack of informative loci) were excluded from these analyses, as well as the nests where the sex ratio of sexual pupae was not obtained (11 cases because of accidental destruction of samples, Table 2).

We estimated genetic relatedness from genotype data using Relatedness 4.2 (Queller & Goodnight, 1989). Worker relatedness gives a reasonable estimate of the conflict structure at the colony level, since it reflects both queen number and relatedness. As queen number and queen relatedness are not correlated in these populations (Hannonen et al., 2004), relatedness between workers and worker-produced males r(wm) declines faster with declining worker relatedness r(w) than does relatedness between workers and queen-produced males r(qm) (Bourke & Franks, 1995). Thus, the incentive for worker reproduction increases and incentive for worker policing decreases with increasing relatedness among workers r(w). Colony level data for r(w) was available only for nests in experiment I and was included in the corresponding GLM model. For experiment II we were only able to test whether the two study populations with different average kin structures differed from each other, thus for experiment II population was included as a variable in the GLM model instead of the colony relatedness values.

Results

Experiment I: The parentage of eggs

In 411 of 624 eggs (65%) we obtained successful amplification at least at some loci. This suggests that 35% of the eggs were either inviable trophic eggs that were laid for nutritional purposes only (Gösswald, 1989) or young eggs that did not contain enough nuclei for successful amplification. On average 2.0 (SD = 0.94) loci per egg amplified successfully. Only eggs that amplified at least at one locus were used in the subsequent analyses. Of the remaining 411 eggs 128 (31%) failed to amplify at the diagnostic locus and could therefore not be assigned to either sex or origin (Fig. 2).

Most of the eggs that amplified at least at one locus were diploid and thus queen-laid females (Table 1, Fig. 2). Worker laid male eggs were found in 15 of the 26 experimental nests (58%), but the average numbers

Table 1 Numbers (mean ± SD) and proportions of female, worker-laid, male and unassigned eggs in Experiment I.

Treatment	Control		Box		Total number	
	Number	%	Number	%	Number	%
Females	14.1 ± 6.3	60.0 ± 31.6	10.5 ± 8.4	53.3 ± 38.4	12.6 ± 7.4	59.5 ± 32.5
Worker-laid males	2.9 ± 3.5	13.5 ± 16.3	2.0 ± 3.0	10.5 ± 16.9	2.5 ± 3.2	12.2 ± 16.3
Unassigned males	1.0 ± 2.4	4.2 ± 10.3	0.8 ± 2.1	2.5 ± 8.3	0.7 ± 2.0	3.5 ± 9.3
Unassigned males and females	3.9 ± 3.3	23.5 ± 26.0	6.3 ± 5.0	33.7 ± 28.9	4.9 ± 4.1	24.7 ± 23.4

**Table 2** Final brood composition among pupae in Experiment II, treatments 1–7.

Nests producing:	Workers			Sexuals		Sons of workers	
Treatment (n)	Mean ratio ± SD	No. of nests	Mean (min, max).	No. of nests	Mean (min, max)	n	Mean (min, max)
1. Queen free (19)	86.4 ± 77.5	16	52.3 (2, 150)	3	8.7 (3, 15)	0	
2. Queen + box (25)	134.2 ± 133.7	24	82.2 (5, 300)	3	9.3 (5, 16)	2/2	1 (1,1)
3. Queen + worker laid eggs (19)	175.5 ± 205.1	16	122.4 (2, 450)	6	4.7 (2, 9)	3/3	3 (1,6)
4. Queen + box + worker laid eggs (14)	213.5 ± 190.8	12	128.2 (5, 300)	3	8.3 (3, 16)	1/1	8
5. No queen + Queen laid eggs (23)	14.3 ± 27.5	5	13.4 (2, 25)	20	23.9 (1, 83)	6/6	9.5 (3, 16)
6. Orphaned (31)	107.9 ± 87.7	20	49.2 (2, 140)	15	19.7 (1, 60)	1/2	5
7. Queen ± Queen laid eggs (10)	264.1 ± 75.4	10	146.7 (89, 220)	0	0	0	

Ratio = ratio of queen vs. worker-produced pupae (±SD). Sample size for nests producing workers' sons is reported as the number of colonies / total number of colonies analysed.

and proportions were low (Table 1, Fig. 2). Although 88% of these classifications were based on one locus and failure rates in DNA amplification were high, these results are unlikely to be biased because of allelic dropout of large alleles for two reasons. First, the overall size range of alleles was very small, the largest size difference between the diagnostic allele and the allele of the queen being only 8 bp. Secondly, in 365 eggs that amplified at multiple loci, all loci gave consistent information.

None of the factors entered in the GLM model (queen confinement, worker relatedness, total production) significantly explained the variation in the number or percentage of worker laid eggs (All $F_{1,14} < 0.28$, n.s.). The fact, that neither the number, nor the percentage of worker-laid male eggs, differed between the control and the box treatment, suggests that queens do not physically interfere with brood composition, and that either chemical control, self policing or worker policing prevails (Table 1). Similarly, the lack of influence of population or worker relatedness on the number and percentage of worker-produced males indicates that workers do not facultatively change their egg-laying behaviour in response to changes in kin structure. Finally, the lack of effect of total production suggests that queen fecundity does not affect worker egg laying behaviour.

Interestingly, the 15 nests where worker-laid eggs were found originated from only seven field colonies out of a total of 18. The highest numbers of worker laid eggs were found in 10 laboratory nests, which originated from two highly polygynous field colonies with five and 50 queens respectively (Fig. 2). In these colonies the
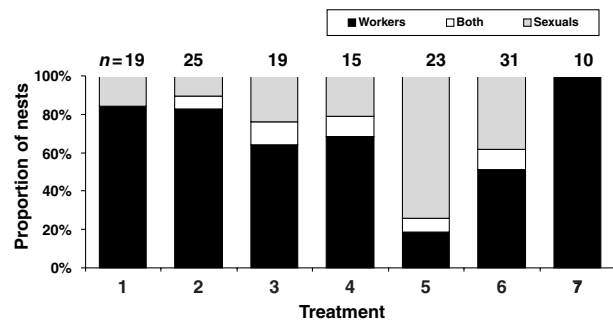
Fig. 3 Percentages of nests rearing workers, sexuals or both in treatments 1–7 (see Fig. 1 and text for explanation of treatments).

average proportions of worker-laid male eggs were 16.9% (SD = 0.10) and 34.8% (SD = 0.17), respectively.

Experiment II: Conflict resolution

The queen right colonies reared mainly new workers (Fig. 3). Only 15 of 88 queen right nests (treatments 1–4 and 7; Table 2) produced any sexual pupae, and only six of these produced worker-derived male pupae (Table 2). On average only 3.17 (SD = 3.1) worker-laid males out of an total average of 92.3 (SD = 84.0) pupae were found in these six nests. This pattern was similar irrespective of queen confinement or egg transfers (Table 2), though we were not able to analyse it formally because of the low

numbers of worker-produced male pupae. Conversely, in the queenless nests which received queen-laid eggs (treatment 5), worker laid males were found in all five nests where males were analysed (mean = 10.5, SD = 4.8).

The proportion of queen right nests rearing workers' sons to pupae [six of 87 laboratory colonies (6.9%)] was lower than the proportion of nests where worker laid male eggs were found [15 of 26 (58%), Fisher's exact test, $P = 0.001$]. The outcome remains significant even if we conservatively assume that also all queenright nests where sexual samples were lost [nine of 87 (10.3 %)] had reared workers' sons (Fisher's exact test, $P = 0.047$). This suggests discrimination against workers' sons.

In the queen right treatments (1–4 and 7) the sex ratio of the few sexuals reared was highly male biased because of worker laid males (mean = 0.91, SD = 0.20, $n = 9$). In the queenless treatments the sex ratio was significantly more male biased in treatment 5 (mean = 0.92, SD = 0.18, $n = 19$) where queen-laid eggs had been introduced into queenless nests after the onset of worker-laying, than in treatment 6 (mean = 0.3, SD = 0.45, $n = 11$), where the queen had been removed (Mann–Whitney $U = 34.5$, $n = 30$, $P < 0.001$). This suggests that when given the choice, workers favoured queen laid female offspring over starting egg-laying by themselves.

In the control treatments, where queen-laid eggs were transferred into queen right nests) 80% of nests reared at least some (mean = 17.8, SD = 10.1) of the transferred offspring. The actual number of transferred offspring reared was probably much higher since the colony queens often shared alleles, which left on average 74% of the pupae unassigned to either queen. Nests receiving queen laid eggs (treatment 7) reared more transferred offspring (mean = 14.3, SD = 11.7) than nests receiving worker laid eggs (treatments 3 and 4 combined mean = 0.52, SD = 1.7, Mann–Whitney $U = 109.0$, $n = 44$, $P < 0.001$), supporting the result that worker laid eggs are policed because of their parentage, not because of the manipulation.

In the presence of a queen, most pupae were queen-produced regardless of queen confinement or egg transfers, whereas more worker-produced pupae were reared in queenless nests where queen-laid eggs had been added (treatment 5) [$F(\text{treatment})_{6,118} = 10.0$, $P < 0.001$; Table 2]. Post hoc comparisons indicated no differences between treatments 1, 2, 3 and 4 (Tukey's test, all $P > 0.99$), but significant differences between treatment 5 and all other treatments (Tukey's test, all $P < 0.001$). This suggests that worker-produced males are reared only in the absence of a queen and her brood but that once the workers have started to lay eggs they cease to accept queen-laid ones. Colonies with a high total production also raised a significantly higher proportion of queen-produced pupae [$F(\text{totalmass})_{1,118} = 95.8$, $P < 0.001$]. The populations did not differ from each other in brood ratio [$F(\text{population})_{1,118} = 0.13$, n.s.].

Discussion

In this study, we have established that some workers actively pursue their selfish interests by laying male eggs but that almost all worker-laid offspring is removed presumably by other workers. As a result broods consist almost exclusively of the queen-produced pupae in both populations studied. Thus our results show that the conflict over male production is expressed, but the low fraction of worker-laid eggs, may indicate a reduction in conflict owing to some degree of self policing. However, as the colonies only reared new workers the cause of male elimination may be either their parentage or their gender.

We found worker laid eggs in the majority of the colonies, although at fairly low numbers. As almost none of the worker-laid eggs were reared into adults in queen-right conditions, and queen confinement had no effect on the outcome, we conclude that workers respond to the presence of a queen by mutual control. This conclusion gains further support from the egg introduction experiments, where most of the additional worker-laid eggs failed to reach maturity. Because nests were checked only once a week to avoid excess disturbance, a large proportion of worker-laid eggs may have been policed prior to sampling. Thus the numbers and proportions of worker laid eggs observed are minimum estimates. In honeybees and ponerine ants workers have been shown to be able to distinguish between queen- and worker-laid eggs at an early stage and remove the latter (Ratnieks, 1993; Monnin & Peeters, 1997; Kikuta & Tsuji, 1999; Monnin & Ratnieks, 2001).

Male elimination prevails in both populations, independent of the population and colony level variation in kin structures. We found no correlation between the proportion of worker-laid eggs and worker relatedness within the populations, and no differences in the proportion of worker-produced pupae between the two populations. This may result from an inability of workers to assess colony kin structure, or lack of incentive to act upon perceived differences. Workers of *F. fusca* are, however, apparently capable of accurate kin discrimination (Hannonen & Sundström, 2003), so lack of incentive seems a more plausible explanation for the absence of facultative egg-laying and brood removal. Indeed, both populations were very close to the threshold value at which workers are indifferent with respect to male parentage. Furthermore, colony kin structure is unstable across years because of queen turnover (Hannonen *et al.*, 2004). As a result the genetic value of worker-produced males will vary between years, which may prevent precise responses. Taken together, the potential benefits from facultative manipulation of male parentage may well be outweighed by costs associated with such behaviour.

In addition to worker policing, self restraint by workers may yield low numbers of worker-laid eggs (Ratnieks, 1988). Self policing independent of relatedness

incentives has often been explained by colony level costs of worker reproduction (Bourke, 1988a; Ratnieks, 1988; Ratnieks & Reeve, 1992; Foster & Ratnieks, 2001; Monnin & Ratnieks, 2001; Tóth *et al.*, 2002a, b, 2004). These costs may result from lower work rates of fertile workers (Cole, 1986; Ratnieks, 1988; Ratnieks & Reeve, 1992; Bourke & Franks, 1995) or recognition errors by workers when replacing queen laid males with their own (Nonacs & Carlin, 1990; Nonacs, 1993). Variation among species in costs and benefits of worker reproduction and policing also partly explains the variation in conflict outcomes mentioned in the introduction.

Self policing is compatible with the idea of queen fertility signalling (Seeley, 1985; Keller & Nonacs, 1993). The higher the reproductive output of the queen the more workers benefit from favouring her offspring instead of their own. Thus, a chemical queen signal conveying reliable information about the fertility of the queen benefits both parties and should be evolutionarily stable (Seeley, 1985; Keller & Nonacs, 1993). Indeed, *F. fusca* workers are able to assess differential queen fecundity based on chemical cues (Hannonen *et al.*, 2002) and our results from queen confinement experiments show that any queen effects must be chemically mediated. Our results support the view of worker self restraint as an adaptive response to the fertility signal of the queen (Seeley, 1985; Keller & Nonacs, 1993; Ortius & Heinze, 1999; Peeters *et al.*, 1999; Liebig *et al.*, 2000) on three accounts. First, workers in orphaned colonies readily laid large numbers of eggs over a short time period, whereas the number of worker-laid eggs always was low in the queen right colonies. Secondly, in orphaned colonies workers raised the queen-laid eggs, whereas workers in queenless colonies raised their own eggs which had been laid prior to the introduction of queen-laid eggs. This suggests that the presence of queen-produced eggs signals queen fertility and prevents worker reproduction. Thirdly, the high number of worker laid eggs in the nest fragments created from two highly polygynous field colonies might be a worker response to low fertility of the queens in these experimental nests. The discrepancy between high observed queen numbers (five and 50 queens) and high worker relatedness ($rw = 0.46$ and 0.58 respectively) implies that only a few queens were reproductively active in these colonies (Pedersen & Boomsma, 1999). The queens in these particular experimental nests were necessarily all mated (except nest 17), as all nests produced diploid offspring. Hence, the workers may have responded to low queen fecundity by increasing egg-laying or decreasing egg policing. Overall, the pattern suggests that self policing, although incomplete, is an important conflict determinant and that workers can flexibly adjust both their egg laying and policing behaviour to the prevailing conditions of queen fertility and presence in the colony. However, our data does not tell what the actual extent of worker egg laying is, as an unknown proportion of the eggs may have been policed before sampling. Thus data about worker ovary development is needed to assess the importance of self policing in *F. fusca*.

Other kin conflicts manifest in the colonies may interfere with the expression of the conflict over male parentage (Walin *et al.*, 1998; Bourke, 1999; Foster & Ratnieks, 2001; Hammond *et al.*, 2003). The same relatedness patterns that predict a conflict over male parentage also predict a conflict over colony sex ratio (Trivers & Hare, 1976; Boomsma & Grafen, 1990, 1991). Thus male offspring may be selectively removed for sex ratio reasons rather than their parentage (e.g. Passera & Aron, 1996; Sundström *et al.*, 1996). In our study colonies very few males were raised in queen-right colonies. This is also consistent with males being removed because of the gender, not their parentage. However, no worker-laid males were found in single-queen field colonies in another population of this species (Olsson, 1999, K. Trontti & L. Sundström, unpublished data), which suggests that any worker-laid eggs are policed in male producing colonies and natural conditions as well. Nevertheless, the fact that workers did lay eggs in the presence of a queen shows that the conflict over male parentage is expressed. With the present data, where male offspring of the queen is very rare, we are not able to distinguish if parentage or sex is the proximate reason for the discrimination against worker laid eggs. We also do not know if queen-laid male eggs are removed, or if the queen lays a female-biased sex ratio. Furthermore, without data on sex ratio variation and worker relatedness towards males and females in the populations we are unable to apply the model of Hammond *et al.* (2003) to the situation. Thus we cannot tell if the conflict over male production or the conflict over sex ratio is the ultimate reason for discrimination.

Two features in the ecology of *F. fusca* may affect the extent of worker reproduction, but in different directions. On the one hand, iteroparity should select for reduced worker reproduction and semelparity for escalation of conflicts (Bulmer, 1981; Bourke, 1994). In iteroparous societies the conflict is not predicted to escalate, because also the workers value the survival of the colony to the next reproductive season. On the other hand, maintenance of worker reproduction may be favoured if the risk of colony orphaning is high. This is probably the case for *F. fusca* as it is a weak competitor (Savolainen, 1990), and frequently subject to social parasitism by other *Formica* species. Thus a certain level of worker fertility may be adaptive as long as the costs because of the expression of the traits are not prohibitive to the colony.

We conclude that workers of *F. fusca* lay eggs in the presence of the queen but these eggs are policed by other workers. Because relatedness incentives do not unequivocally predict worker policing, we suggest that colony level costs of worker reproduction favour worker policing. Worker policing works to maintain colony

cohesion and reduces the incentive for selfish worker reproduction. Future studies should assess colony level costs of worker reproduction and the role of self policing in conflict resolution.

Acknowledgments

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