The value of oviposition timing, queen presence and kinship in a social insect

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Reproductive cooperation confers benefits, but simultaneously creates conflicts among cooperators. Queens in multi-queen colonies of ants share a nest and its resources, but reproductive competition among queens often results in unequal reproduction. Two mutually non-exclusive factors may produce such inequality in reproduction: worker intervention or queen traits. Workers may intervene by favouring some queens over others, owing to either kinship or queen signals. Queens may differ in their intrinsic fecundity at the onset of oviposition or in their timing of the onset of oviposition, leading to their unequal representation in the brood. Here, we test the role of queen kin value (relatedness) to workers, timing of the onset of oviposition and signals of presence by queens in determining the maternity of offspring. We show that queens of the ant *Formica fusca* gained a significantly higher proportion of sexuals in the brood when ovipositing early, and that the presence of a caged queen resulted in a significant increase in both her share of sexual brood and her overall reproductive share. Moreover, the lower the kin value of the queen, the more the workers invested in their own reproduction by producing males. Our results show that both kinship and breeding phenology influence the outcome of reproductive conflicts, and the balance of direct and indirect fitness benefits in the multi-queen colonies of *F. fusca*.

1. Introduction

Breeding in a group may confer greater fitness returns than solitary breeding, owing to synergy effects through collaboration [1–3]. However, as limited resources may constrain group productivity, not all individuals in the group may realize their full breeding potential, and this introduces a potential conflict between breeders. Competition for a larger share of reproduction may then lead to an actual conflict over reproduction among individuals [4].

In social insects, several reproductive females (queens) commonly share a colony and the resources it represents (polygyny) [5,6]. Polygyny may confer a range of fitness benefits at both the colony and the individual level, such as increased parasite resistance through greater genetic diversity [7] and increased colony longevity [8]. At the same time, polygyny creates potential conflicts among queens, between workers and queens, and among workers [9]. On the one hand, queens may come to compete for reproductive shares, with unequal reproduction as a result [10–14], and on the other hand, the presence of multiple matrilines dilutes the average kin value (relatedness) of brood to the workers. This creates an incentive for workers to modify brood composition to their genetic advantage [15].

Queens may affect their reproductive share directly through dominance interactions, or indirectly through changes in brood composition mediated by workers. In the latter case, worker behaviour may be influenced by the queens; for example, a queen may signal her presence and fecundity to workers, and so gain their attention [16]. In addition to within-colony interactions, external factors may influence the sharing of reproduction. In particular, seasonality limits the time available for reproduction, which should favour an early onset of oviposition, provided queens are fed adequately. Given that in boreal ants sexuals are usually reared from the first brood cohort of the breeding season...
[17], the ecological incentive for queens to initiate oviposition early becomes even stronger.

Worker and queen interests may be aligned, if queens with a high early fecundity also are of greater kin value to the workers. If this is not the case, workers and queens may come into conflict over reproductive shares, especially if the early eggs have a higher likelihood of developing into sexual brood. This raises the issue over who controls brood maternity, the queens or the workers. As the number of queens increases, the average genetic value of queens and their sexual offspring to workers decreases. This creates an incentive for the workers to start laying male-dominated haploid eggs [18]. In our study species, the black ant *Fornica fusca* (Linnaeus 1758), multiple queen and unequal reproduction are common [10], and workers readily start laying male eggs upon orphaning [19].

The objectives of our study were to use a combination of laboratory experiments and genetic parentage assignment to disentangle effects of queen presence and oviposition date on key components of worker and queen inclusive fitness. Following the argument by Keller & Nonacs [20] that workers benefit in terms of their own inclusive fitness by responding to a queen signal, we hypothesize that by signalling her identity to workers a queen may gain a larger share of reproduction. Similarly, queens that initiate egg-laying early in the season may gain a greater share of sexual production, given that the first cohort of brood regularly develops into sexuals in *Fornica* species [17,21,22]. Conversely, workers may replace early queen eggs when the kin value of the queen is low, and so maximize their own inclusive fitness. To achieve our objectives, we manipulated the onset of oviposition and queen presence in laboratory nests in two experiments, and compared queen reproductive shares and the magnitude of worker reproduction upon brood pupation.

### 2. Material and methods

#### (a) Field collection and colony maintenance

Entire colonies of *F. fusca* were collected in April 2008, at the end of hibernation but before the onset of egg laying, in southwest Finland, on the Hanko peninsula, near Tvärminne zoological station. The colonies were brought to the laboratory and sorted, and colonies with two queens were selected for the experiments. The colonies were then placed in open plastic trays, the walls of which were coated with Fluon. The trays were lined with peat, and kept in darkness at +4 °C until the start of the experiments to standardize the onset of oviposition across colonies.

#### (b) Experiment 1: effect of onset of oviposition on queen reproductive share

In the first experiment, we tested whether the onset of egg-laying affects the reproductive share queens obtain by introducing eggs laid by two queens into the same nest 6 days apart. To obtain queen-laid eggs, we divided each field colony into four fragments: two with approximately 70 workers and one queen each to produce eggs for the experiment (‘egg-production fragments’), and two others with approximately 250 workers. These were the orphaned experimental nests, which received eggs laid in the egg-production fragments (see the electronic supplementary material, figure S1). The egg-production fragments were housed in smaller trays (8 × 10 × 8 cm) than the experimental fragments (30 × 40 × 15 cm); the latter also contained a bathroom tile (15 × 15 cm) as shelter.

To start the experiment, we transferred the egg-production fragments from +4 °C to 25°C and checked them every 72 h for the presence of eggs. Once the eggs were present in these fragments, the corresponding recipient nests were also transferred from +4 °C to 25°C. Then, eggs laid by the two queens were transferred into the two experimental nests (see the electronic supplementary material, figure S1, ‘orphaned nests’). If not enough eggs were obtained during the first transfer, the procedure was repeated within 3 days until altogether 50 eggs had been transferred into each recipient nest. Six days after the first set of 50 eggs had been transferred, the introduction of the second set started (see the electronic supplementary material, figure S1). The workers were then allowed to rear the brood until pupation. The recipient nests were maintained at 23–27°C, fed Bhatkar & Whitcomb diet [23] and moistened daily.

#### (c) Experiment 2: effect of queen presence on her reproductive share

In this experiment, we used a different set of colonies and tested whether the presence of a queen affected the rearing preferences of the workers. We tested this in a setting that also included the effect of precedence in egg-laying. Thus, the experimental set-up and procedures followed those in experiment 1, except that one of the queens was present inside the recipient nest, but caged so that the workers were denied access to her or the eggs she was laying during the experiment. The cage had openings on the top and the front side, each 8 × 2 cm in dimension and covered with a single layer of cloth mesh (hole width 0.5 mm) precluding any possibility of tactile contact. The cage (8 × 10 × 8 cm) with the queen and approximately 70 workers was placed in the recipient nest once the egg introduction was completed (see the electronic supplementary material, figure S1, ‘queen-right nests’). In half the replicates, the queen whose eggs were introduced first was placed in the nest, and in half the replicates, the queen whose eggs were introduced second was placed in the nest. The queens were assigned to their role randomly. In total, 14 and 13 different polygyne colonies were used for experiment 1 and experiment 2, respectively.

#### (d) Genotyping and parentage assignment of offspring

Once the brood had pupated after approximately five weeks, all the pupae were classified to caste—male, young queen (‘gynae’) or worker—based on their morphology, and, together with the mother queens and a random sample of eight workers per colony, were killed in 94% ethanol and genotyped (see the electronic supplementary material, methods S1). To assign the maternity of brood, we identified for each pair of queens one or more diagnostic loci at which either the mother queens or the sperm in their spermatheca differed, and assigned all diploid brood accordingly to one of the two queens. Males can be produced by either queens or workers in this species [19], and by the same principle, we assigned all males that carried an allele absent in both queens as worker-produced; otherwise, the maternity of the male was considered inconclusive (data on queen shares and worker reproduction are available at Dryad doi:10.5061/dryad.h7km8).

#### (e) Statistical analyses

We first applied data exploration techniques following the protocol of Zuur et al. [24] and then used generalized linear mixed models (GLMMs) on untransformed data using the package lme4 in R [25,26]. First, we compared total brood production and caste ratios across all colonies in a model with queen presence/absence, brood type (male, gynae and worker) and their interaction as fixed factors, and colony as a random factor.
using GLMM with Poisson errors. The response variable was the number of queen-derived offspring reared in each colony. We treated data overdispersion (residual deviance: d.f. = 8.82) by adding the individual-level random variable to create the final model [27]. We then tested for the effect of queen precedence in oviposition on her brood share in the absence (experiment 1) and the presence of a queen (experiment 2) using GLMM with binomial errors. The models included either queen oviposition order only (experiment 1) or queen oviposition order, queen presence and their interaction (experiment 2) as fixed factors, and colony and queen identity as random factors to account for repeated data [28]. In both cases, the response variables were the total number of offspring and the number of sexual offspring attributed to each queen out of the 50 eggs introduced in the experiment. We used the number of sexual offspring, rather than sex ratios, as males and females are of equal kin value to the queen, and thus best reflect their reproductive success. We tested for significant effects of the fixed factors and/or their interaction with the likelihood-ratio-based $\chi^2$-test (LRT). In addition, we estimated both average within-colony relatedness (i.e. calculated across colonies), and the pairwise relatedness between queens and workers based on genotype data using RELATEDNESS v. 5.0.4 [29]. We then used Pearson’s product–moment correlation to examine the effect of queen–worker relatedness on total and sexual brood production, and an ANCOVA to assess the effect of queen–worker and worker–worker relatedness on the production of queen-versus worker-produced males, respectively.

### 3. Results

Altogether, the experimental nests ($n = 27$) produced on average $14.4 \pm 17.1$ (mean $\pm$ s.d.) worker and $14.3 \pm 7.9$ sexual (gyne and male) pupae distributed across 17 and 26 nests, respectively (see the electronic supplementary material, table S1). The fraction of colonies that produced sexuals was high, because we targeted the cohort that normally would include sexual brood. There was no significant difference in productivity in terms of either the total number of offspring or the number of offspring of each brood type produced in orphaned versus queen-right fragments (LRT: queen presence/absence, $\chi^2 = 0.14$, d.f. = 1, $p = 0.705$; brood type, $\chi^2 = 8.45$, d.f. = 2, $p = 0.015$; queen presence/absence $\times$ brood type, $\chi^2 = 3.51$, d.f. = 2, $p = 0.173$). In total, 277 gynes and 103 males were produced, yielding an average colony sex ratio that was female-based at $0.75 \pm 0.32$. The maternity of 41 gynes and two males could not be unambiguously assigned to either one of the two experimental queens or queen and worker, so these were later excluded from the analyses.

In the first experiment, in which neither of the queens was present, the order of introduction of the eggs had no significant influence on the total number of offspring reared per queen (LRT: $\chi^2 = 0.02$, d.f. = 1, $p = 0.898$; figure 1a). However, when only sexual offspring were considered, the queen whose eggs were introduced first had a significantly greater likelihood of having her offspring (gynes and males) reared to adulthood than the queen whose eggs were introduced later (LRT: $\chi^2 = 31.06$, d.f. = 1, $p < 0.001$; figure 1b). In the second experiment, in which one of the queens was present in a cage in the nest during brood rearing, egg order did not significantly affect the brood share of each queen (LRT: $\chi^2 = 0.01$, d.f. = 1, $p = 0.918$). However, the queen that was present in a cage gained a significantly greater overall share of the total brood than the absent queen (LRT: $\chi^2 = 47.56$, d.f. = 1, $p < 0.001$). Moreover, the queen with a delay in oviposition benefited more by being present during brood rearing than the queen with early oviposition, as indicated by a significant interaction between the egg order and queen presence (LRT: $\chi^2 = 32.19$, d.f. = 1, $p < 0.001$; figure 1c). When only sexual offspring were considered, both egg order and queen presence significantly affected queen reproductive share, so that the queen whose eggs were introduced earlier, and who was present during brood rearing, gained significantly more sexual offspring than the queen whose eggs were introduced later or who was absent (LRT: egg order, $\chi^2 = 5.04$, d.f. = 1, $p = 0.025$; queen presence/absence, $\chi^2 = 8.08$, d.f. = 1, $p = 0.004$). Moreover, the significant interaction between egg order and...
queen presence suggests that queen presence reinforces the positive effect of early oviposition on the number of sexual offspring gained (LRT: $\chi^2 = 8.94$, d.f. = 1, $p = 0.003$; figure 1d).

The average relatedness among queens and workers in our experimental nests was 0.56 ± 0.29 and 0.29 ± 0.14 (mean ± s.d.), respectively. The average queen kin value to workers (queen–worker relatedness) was 0.36 ± 0.18 (mean ± s.d.), and affected neither total offspring production ($r = -0.01$, d.f. = 25, $p = 0.98$) nor sexual production significantly ($r = -0.18$, d.f. = 25, $p = 0.37$; electronic supplementary material, figure S2). Male production, however, increased significantly with decreasing queen kin value (colony-specific queen–worker relatedness) to workers ($r = -0.51$, d.f. = 23, $p = 0.01$; figure 2). More importantly, of the 101 males that were produced in the 14 male-producing nests, 75 were worker-produced, whereas the genotypes of the remaining 26 males were consistent with those of at least one of the queens, and hence further considered as queen-produced. When males were separated according to maternity the fraction of worker sons decreased significantly with increasing queen kin value, whereas no such change was found for queen sons (ANCOVA: queen–worker relatedness, $F_{1,24} = 6.16$, $p = 0.021$; male maternity, $F_{1,24} = 6.15$, $p = 0.021$; queen–worker relatedness × male maternity, $F_{1,24} = 4.45$, $p = 0.046$; figure 2). Although the production of worker sons also decreased with increasing worker relatedness, neither this association nor the interaction between male maternity and worker–worker relatedness were significant (worker–worker relatedness, $F_{1,24} = 3.38$, $p = 0.079$; male maternity, $F_{1,24} = 5.29$, $p = 0.031$; worker–worker relatedness × worker origin, $F_{1,24} = 2.41$, $p = 0.134$; electronic supplementary material, figure S3).

4. Discussion

In this study we tested inclusive fitness implications of co-breeding for both queens and workers in the ant F. fusca. We show that queens with an early onset of oviposition gained substantial fitness benefits by obtaining the majority share of sexual production, and that a caged queen can influence brood composition in her favour, most probably via odour cues. Thus, queens can influence their reproductive success by adjusting their timing of oviposition, and by communicating their identity to the workers, so influencing the composition of the brood the workers raise. The timing of oviposition may be indirectly influenced by the workers through differential treatment of queens. Conversely, when queen kin value was low, some workers started to lay male-destined eggs. Thus, workers can on their part assess their indirect fitness benefits from rearing queen-produced brood, and at least some workers shift towards increased direct (rather than indirect) fitness returns, when the genetic returns from raising queen-laid brood dwindle. Taken together, our results demonstrate that the different parties within a social insect colony can simultaneously gain inclusive fitness returns while acting in their own genetic interests.

Given that eggs laid early in the season more often develop into sexuals, whereas those laid later develop into workers, queens who start ovipositing early stand to gain considerable fitness returns. Since we used a reciprocal design to account for potential differences between the eggs laid by different queens, the observed pattern probably reflects the differential treatment of brood by workers. The benefits to queens are therefore indisputable, but the question arises as to what inclusive fitness benefits workers derive from this. An earlier study on F. fusca showed that within each year the adult workers and the new queens they raise in spring were the offspring of the same subset of the total pool of reproductive queens in the colony [30]. Therefore, the workers are particularly closely related to the sexuals they help raise, which secures an unambiguous inclusive fitness increment to the contemporary workforce. Thus, fitness gains may emerge simultaneously for both queens and workers.

In addition to an early onset of oviposition, the opportunity for a queen to communicate her presence also conferred fitness advantages. In particular, the queen in situ had a greater proportion of her eggs reared in total and as sexuals, regardless of the order in which the eggs were introduced. Thus, queen presence reinforced the positive effect of early onset of oviposition on sexual brood production and, in addition, increased the total reproductive share of the queen with oviposition delay. Two conclusions follow from the fact that the presence of a queen affected her reproductive share. First, queens apparently communicate their presence, and possibly their fecundity, to workers, which subsequently influences the brood-rearing decisions workers make. Recent studies suggest that non-volatile hydrocarbons present on the cuticle of adult individuals and/or eggs may act as queen pheromones [31–33], which can be perceived by workers at short distances in the absence of direct contact [34,35]. Thus, the workers could have perceived queen odours through the mesh. Alternatively, cues may have been emitted via volatile compounds, which have been implicated as queen pheromones in social insects other than ants [36–38]. However, none of the studies to date have found evidence for volatile queen pheromones affecting reproduction in ants [39–41]. Second, our study shows that workers combine chemical information on queen identity and their eggs, and act upon this information by favouring the eggs of the present queen. It seems that workers are able to incorporate cues of queen identity into their recognition template [42], which subsequently improved the acceptability of eggs laid by the caged queen. This reinforces earlier results that have suggested unusually precise recognition abilities of F. fusca workers [15,43], and suggests that queen eggs carry recognition cues.

Finally, workers reared more of their own sons when queen kin value declined. This suggests that F. fusca workers can assess the kin value of either the brood or the queen(s).
Thus, workers increase their inclusive fitness by trading indirect fitness gains from rearing queen brood for direct fitness gains from own reproduction as their relatedness to queen brood falls [18,44]. Similar facultative variation in worker reproduction has been demonstrated only in the vespine wasp Dolichovespula saxonica [45] (but see [46]). Our results provide solid new evidence for inclusive fitness-driven brood manipulation, and suggest that queen or brood kin value may better measure net inclusive fitness returns from worker reproduction than relatedness among workers (cf. [47]). This may be the case especially in polygynous species with rapid queen turnover, like F. fusca [30,48]. Hence, contemporary workers are not necessarily the offspring of queens currently present in the colony, but at least partly represent the offspring of queens that have died. When the workers and the brood being raised only partly have the same parents, the kin value of currently breeding queens may better reflect the average indirect fitness returns to workers than the average relatedness among workers.

The balance of direct and indirect fitness benefits of F. fusca workers parallels patterns in cooperatively breeding vertebrates, where the degree of flexibility and discrimination in helping decisions has been shown to predominantly occur in species with intermediate and highly variable relatedness within groups [49]. First, rearing of early eggs into sexuals seems to occur without active kinship assessment, and indirect fitness benefits via this route seem on average high ([30] and this study), so that discrimination is not favoured by selection. A similar example of kinship benefits through passive processes has also been described in a Formica species where discrimination abilities are very poor [50]. Second, earlier studies have shown that where workers face highly variable indirect fitness returns from offspring of different queens in an experimental setting, they increase their indirect fitness through nepotistic brood rearing [15]. Even if such nepotism is predicted to be rare in social insects [51], the intermediate and variable relatedness within F. fusca nests combined with maintenance of high chemical cue diversity facilitated by social parasitism [52] could provide a setting for nepotistic discrimination. Third, workers adjust the balance of helping and individual reproduction in response to the average kin value of brood (this study), and opt for direct fitness when closely related brood is not available at all. Such reproductive adjustments are common in vertebrates [49], but examples have been rare in social insects.

We suggest that further social insect examples might be found in species such as F. fusca, where relatedness is intermediate and variable, and hence the benefits of kin discrimination are potentially great.

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