

# Unequal partitioning of reproduction and investment between cooperating queens in the fire ant, *Solenopsis invicta*, as revealed by microsatellites

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## SUMMARY

Social insects provide ideal systems for investigating how kinship and ecological factors affect cooperation and conflict. In many ant species, unrelated queens cooperate to initiate new colonies. However, fights between queens break out after the eclosion of the first workers, leading to the death of all but one queen. Queens within associations potentially face a trade-off. On one hand, a queen should restrain her investment in brood production and care if this helps her to maintain fighting ability. On the other hand, a queen may benefit by increasing her contribution to brood production if having more daughter workers than her cofoundresses enhances her chances of taking over the colony. Increased investment is also beneficial because a large brood enhances colony survival. Using microsatellites, we determined the maternity of workers (adults and larvae) at the time of queen execution in the fire ant, *Solenopsis invicta*. Differential mass loss by initially equal nestmates affected survival, with the queen losing less body mass being more likely to survive. Surprisingly, the queen which lost less body mass, that is the one which provided the lowest energy investment, was the one which achieved higher maternity. A control experiment indicated that interactions among queens are responsible for this differential partitioning of reproductive and investment tasks between nestmates. The finding that the queen most likely to win the fights is the one with above-average maternity may explain why workers apparently do not attempt to influence the outcome of fights.

## 1. INTRODUCTION

Although cooperation entails many potential benefits for group members, conflicts almost invariably occur within groups of non-clonal organisms (Seger 1991). Social insects have emerged as a useful system to investigate the dynamics between cooperation and conflict within groups (Bourke & Heinze 1994; Keller & Reeve 1994; Sundström 1994; Bourke & Franks 1995; Evans 1995; Reeve & Keller 1995, 1997; Crozier & Pamilo 1996; Sundström *et al.* 1996), and colony founding by multiple queens provides a particularly simple model for such studies (Hamilton 1964; Bartz & Hölldobler 1982; Rissing & Pollock 1988; Seger 1993; Sommer & Hölldobler 1995; Balas & Adams 1996; Bernasconi & Keller 1996). The founding of new colonies is a critical stage in the life cycle. In most ant species, colonies are founded after nuptial flight by queens without worker assistance. The queen seals herself in a burrow and rears her first workers entirely on metabolic energy stores (Keller & Passera 1989; Wheeler & Buck 1995). For instance in the fire ant, *Solenopsis invicta*, queens lose

about 50% of their initial mass from the beginning of colony founding to worker eclosion three weeks later (Tschinkel 1993; Bernasconi & Keller 1996). Most young colonies perish early due to competition with other recently-founded nests, which is mediated by the brood-raiding behaviour of the first workers (Bartz & Hölldobler 1982; Rissing & Pollock 1988; Tschinkel 1992). Queens of several species (Rissing & Pollock 1988), including the monogyne form (i.e. mature colonies contain only one queen) of the fire ant *S. invicta* (Markin *et al.* 1972), can either embark on colony founding alone or join other queens from the same mating flight to initiate a colony. Cofoundress relatedness was not significantly different from zero in two species investigated (Hagen *et al.* 1988), and this is likely to be a general feature of ant founding associations (Bourke & Franks 1995). Foundress associations produce a larger first worker brood than solitary foundresses (Tschinkel & Howard 1983). This increases their competitive ability and results in higher colony survival and faster maturation (Rissing & Pollock 1988, 1991; Vargo 1988; Adams & Tschinkel 1995).

Although cooperation increases colony survival, it is a high-stakes gamble for individual queens (Seger 1993). In most species, after worker eclosion aggressive

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behaviour breaks out among colony members, until all but one queen are killed or expelled (Wilson 1966; Rising & Pollock 1988; Heinze 1993; Sommer & Hölldobler 1995). The surviving queen monopolizes colony reproduction, while losing queens have zero fitness, since no sexual offspring are produced at this stage.

The survival prospects of queens within *S. invicta* associations have been shown to be associated with their differential mass loss during the founding stage. In pairs of queens of the same (Bernasconi & Keller 1996) or random (Balas & Adams 1996) initial mass, the queen losing less mass up to worker eclosion was more likely to survive. Mass loss is a likely measure of the queens' investment into brood production, as it is positively correlated with worker production in one- (Tschinkel 1993) and two-queen colonies (combined mass loss of both foundresses; Tschinkel 1993; Balas & Adams 1996; Bernasconi & Keller 1996). Thus, queens within associations potentially face a trade-off. On one hand, a queen should restrain investment if this helps her to maintain fighting ability (Reeve & Ratnieks 1993; Balas & Adams 1996; Bernasconi & Keller 1996). On the other hand, a queen benefits by increasing her contribution to brood production, because a large brood enhances survival at the colony level (Bourke & Franks 1995).

An additional factor to be considered in relation to the outcome of the conflict between queens is workers' genetic interests. Since queens are most likely unrelated, the inclusive fitness of individual workers depends entirely on which of the queens survives. The workers might favour their mother if they can recognize her, either through genetic cues or, alternatively, by identifying the most productive queen. On average, the probability that a worker descends from one given queen is higher for the most productive queen (Forsyth 1980). Previous studies did not support the hypothesis that fire ant workers can use genetic cues to recognize and favour their mother (Balas & Adams 1996; Bernasconi & Keller 1996). Although fire ant workers have been observed to attack queens (Balas & Adams 1996; Bernasconi & Keller 1996), the available evidence suggests that direct queen–queen aggression plays the major role in determining which queen will survive (Balas & Adams 1996; Bernasconi & Keller 1996). Thus, it is of interest to know how the productivity of cofounding queens is associated with survival. If the higher investment of one queen results in greater maternity but lower survival probability, this may conflict with workers' interests, since the mother of the most numerous workers would be most likely to die. In their study, Balas & Adams (1996) found no significant relationship between maternity and survival, but their result is based on a small sample (14 colonies), giving a low information content for the logistic regression analysis which they used.

The present study was designed to test whether differential mass loss within queen pairs reflects the number of worker offspring produced by each queen and whether relative maternity is associated with queen survival. The maternity of the offspring present in the colony at the time when one of the queens was killed in fights was assessed for two developmental stages

Table 1. Mean proportion ( $\pm$ s.d.) of the combined mass loss and of the offspring due to the queen losing less mass for the two collection sites ( $U_{min}$  = collection sites compared with Mann–Whitney  $U$ -test)

population	Georgia	Florida	
loss of mass	0.44 $\pm$ 0.05	0.45 $\pm$ 0.04	$U_{min} = 71.0 p > 0.20$
offspring	0.55 $\pm$ 0.19	0.62 $\pm$ 0.15	$U_{min} = 63.5 p > 0.20$
adult workers	0.54 $\pm$ 0.22	0.59 $\pm$ 0.14	$U_{min} = 63.5 p > 0.20$
worker larvae	0.60 $\pm$ 0.28	0.71 $\pm$ 0.17	$U_{min} = 62.0 p > 0.20$
number of cases	18	9	

(adult workers and worker larvae), using microsatellite DNA markers.

## 2. MATERIAL AND METHODS

Queens were collected after two mating flights in early summer 1995 from populations in Georgia and Florida (USA). These populations belong to the monogyne form, i.e. mature colonies contain only one queen (Ross & Fletcher 1985a) and colony founding occurs as described, with either solitary or multiple (2–10) queens (Markin *et al.* 1972; Tschinkel & Howard 1983). Queens were placed in laboratory nests (see Bernasconi & Keller 1996) within 48 h of collection. This is referred to as day 1. In each nest we placed two queens of equal initial mass ( $\pm 0.1$  mg; precision balance Mettler AE100 (Greifensee/CH)), individually paint-marked on the thorax. The colonies were housed in a dark, ventilated chamber at  $29 \pm 2^\circ\text{C}$ ,  $70\% \pm 10\%$  relative humidity ( $x \pm$  range). We reweighed the queens on day 21, immediately before eclosion of the first worker cohort. Starting on day 21, colonies were fed with homogenized beef meat every other day, and monitored daily until one queen was killed. All colony members were then immediately stored in 70% ethanol in separate tubes. At queen execution, the colonies had  $30 \pm 11$  adult workers, and workers in younger developmental stages ( $7 \pm 5$  pupae,  $11 \pm 13$  large larvae,  $11 \pm 11$  medium-sized larvae and  $10 \pm 7$  small larvae;  $x \pm$  s.d.,  $n = 27$ ).

Kinship analysis using microsatellite DNA was carried out for 27 two-queen colonies, 18 from Georgia and 9 from Florida. Because there was no significant difference between the collection sites in the variables measured (mass loss and maternity; table 1), we present combined data. In each colony we genotyped both queens, their spermathecal contents, and a sample of brood ( $21 \pm 4$  adult workers and  $7 \pm 2$  small worker larvae;  $x \pm$  s.d.,  $n = 27$ ). We selected these two developmental stages to address temporal stability of the queens' genetic representation in the progeny. Queen execution occurred 5–26 (median: 11) days after worker eclosion. As the egg stage lasts five days and the total immature period 21 days, the fraction of small larvae reflects oviposition at about the time of worker emergence, while the fraction of adult workers reflects oviposition during the first week of colony founding (Voss & Blum 1987). We genotyped larvae rather than eggs because amplification of egg DNA was frequently unsuccessful, possibly due to the occurrence of trophic (Voss & Blum 1987) or very young eggs with insufficient DNA. Individual ants were homogenized in 1.5 ml tubes with a pestle and digested for two hours at  $55^\circ\text{C}$  in 150  $\mu\text{l}$  extraction buffer (100 mM NaCl, 50 mM Tris-HCl (pH 8), 1 mM EDTA, 0.5% SDS and 200  $\text{mg ml}^{-1}$  proteinase K). After phenol-chloroform

Table 2. *Relative mass on day 20 and maternity of the surviving queens compared to their nestmates (27 colonies)*

surviving queen	relative contribution to workers (adults+larvae)			relative contribution to workers (adults)			relative contribution to workers (larvae)			percentage
	more	less	equal	more	less	equal	more	less	equal	
heavier	15	5	1	12	8	1	17	3	1	78
lighter	3	2	1	3	3	0	2	4	0	22
percentage	67	26	8	55	41	4	70	26	4	

extraction the DNA was precipitated with 1:10 volume 3 M sodium acetate and 2.5 volumes absolute ethanol. The pellets were rinsed with 70% ethanol and dried at 37 °C. Sperm was obtained by dissecting queens, transferring each spermatheca to a petri dish with 150 µl of extraction buffer, puncturing it and collecting the mixture of released sperm and buffer simultaneously with a pipette. Sperm DNA was extracted as described above. We resuspended DNA from queens in 200 µl of distilled water, and that from sperm and workers (adults and larvae) in 100 µl of distilled water.

To amplify microsatellite loci, we used four primer pairs (*Sol11*, *Sol42*, *Sol49*, *Sol55*). The description and sequence of these primers will be published elsewhere (Krieger & Keller 1997). The sequences are available from the authors upon request or through the GenBank database (accession nos. AF002230, 33, 34 and 36, respectively). We set up PCR reactions in 10 µl of reaction mixture containing PCR buffer (Eurobio), 1 mM MgCl<sub>2</sub>, 75 µM dCTP, dGTP and dTTP, 7 µM dATP, 0.2 µg µl<sup>-1</sup> BSA, 0.5 mM of each primer, 1 µl (about 10 ng) of template DNA, 0.02 µl of <sup>33</sup>P-dATP and 0.5 U of Taq polymerase (Extra-Pol II, Eurobio). We amplified all loci using the same standardized cycling profile on Perkin-Elmer (Norwalk, CT, USA) and Biometra (Göttingen, D) thermocyclers: initial denaturation step at 92 °C (2 min), followed by 35 cycles at 92 °C (45 s), 55 °C (45 s) and 72 °C (1 min), and one final elongation step at 72 °C (5 min). Samples of PCR products (1 : 1.5 dilution) were resolved on polyacrylamide sequencing gel (6% acryl-bisacrylamide, 8 M urea). Assignment of maternity from genetic data is simple because queens mate with a single male (Ross & Fletcher 1985a) and males are haploid. Paternal genotype was inferred from sperm in the queen's spermatheca. Maternity was assigned with *Sol11* in 14 colonies, *Sol49* in 12 colonies and *Sol55* in one colony. An additional colony was discarded because none of the four markers allowed to assign maternity.

To study the relationship between mass loss and brood production in the absence of interaction between queens, we monitored queen mass loss and quantified brood production in 70 single-queen colonies kept under the same conditions as described above. Colonies with diploid males (Ross & Fletcher 1985b) or where the queen died ( $n = 26$ ) were excluded from analysis. Brood production was quantified as the number of pupae present on day 21 (previously found to be positively correlated with the number of adult workers present at queen execution; Bernasconi & Keller 1996). To obtain a measure allowing comparison of one- and two-queen colonies, we paired the values for each of two singly-kept queens of the same initial mass. For each pair of values we calculated the relative contribution of the two queens to the combined mass loss and to the combined brood production. For both control pairs of one-queen colonies and for two-queen colonies, these measures are expressed as the proportion attributable to the queen losing less mass.

Means are followed by  $\pm$ s.d.;  $p$ -values are two-tailed;  $r_s$  indicates the Spearman rank correlation coefficient.

### 3. RESULTS

In two-queen associations the queen losing less mass had significantly more worker larvae ( $4.7 \pm 2.3$ ) than her nestmate ( $2.4 \pm 1.6$ , Wilcoxon signed rank test:  $z = -2.9$ ,  $n = 27$ ,  $p < 0.01$ ), but not significantly more adult workers (Wilcoxon signed rank test:  $z = -1.5$ ,  $n = 27$ ,  $p = 0.13$ ), although the difference was in the same direction ( $11.2 \pm 3.7$  versus  $9.4 \pm 4.2$ ). The proportion of worker larvae and of adult workers due to the queen losing less mass were significantly positively correlated ( $r_s = 0.44$ ,  $n = 27$ ,  $p < 0.05$ ), indicating that the apportionment of maternity between queens was temporally stable. Overall, the queen losing less mass had significantly more workers (adult and larvae;  $15.8 \pm 5.3$ ) than her nestmate ( $11.8 \pm 4.7$ , Wilcoxon signed rank test:  $z = -2.2$ ,  $n = 27$ ,  $p = 0.03$ ).

Differential mass loss from day 1 to day 21 was significantly associated with the chance of survival (mass loss in % of the initial mass: survivor  $36.4 \pm 6.6\%$  and loser  $42.7 \pm 4.8\%$ ; Wilcoxon signed rank test:  $z = 3.03$ ,  $n = 27$ ,  $p = 0.002$ ). The *surviving* queen on average had more worker offspring than her nestmate ( $15.6 \pm 5.3$  versus  $12.0 \pm 4.9$ ; Wilcoxon signed rank test:  $z = -1.9$ ,  $n = 27$ ,  $p = 0.05$ ), a result consistent with the finding that differential mass loss was associated with both maternity and survival. Table 2 gives the frequency of colonies in which the surviving queen was both heavier and had more worker offspring than her nestmate.

Across colonies there was a significant, *negative* relationship between the proportion of total colony mass loss due to the queen losing less mass and the proportion of worker offspring due to this queen ( $r_s = -0.52$ ,  $n = 27$ ,  $p < 0.02$ ). This correlation was significant and negative for both the proportion of adult workers ( $r_s = -0.41$ ,  $n = 27$ ,  $p < 0.05$ ) and of worker larvae ( $r_s = -0.39$ ,  $n = 27$ ,  $p < 0.05$ ). Thus, the smaller the relative investment by the queen losing less mass, the higher her relative share in the brood (figure 1a). The combined absolute mass loss of both queens was significantly positively correlated with the total number of workers produced ( $r_s = 0.41$ ,  $n = 27$ ,  $p < 0.05$ ) but not significantly correlated with the proportion of the combined mass loss due to the queen losing less mass ( $r_s = 0.21$ ,  $n = 27$ ,  $p > 0.20$ ).

Control pairs of solitary queens and two-queen colonies were homogeneous with respect to the relative mass loss of queens (table 3). In control pairs of solitary queens, the queen losing less mass produced significantly fewer pupae ( $11 \pm 9$ ) than the other queen ( $20 \pm 6$ ; Wilcoxon signed rank test:  $z = 3.5$ ,  $n = 22$ ,

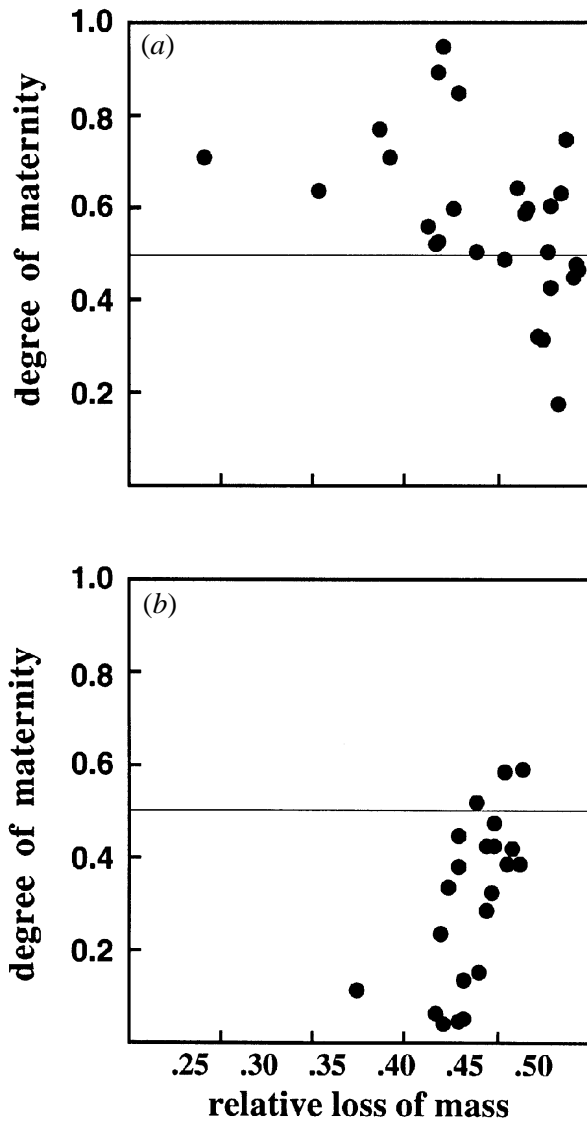


Figure 1. Relationship across colonies between the proportion of offspring (degree of maternity) achieved by the queen losing less mass, and her relative loss of mass, calculated as a proportion of the combined mass loss of the two queens: (a) two-queen colonies; (b) paired values of solitary queens (control).

$p = 0.001$ ; table 3). The relationship between the proportion of mass loss and the proportion of worker offspring was positive across pairs of solitary queens of the same initial mass ( $r_s = 0.65$ ,  $n = 22$ ,  $p < 0.002$ ; figure

1b). This correlation coefficient is of opposite sign and significantly different from the correlation coefficient found for two-queen colonies ( $z$ -test after Fisher  $z$ -transformation:  $z = -4.5$ ,  $n_1 = 22$ ,  $n_2 = 27$ ,  $p < 0.002$ ; table 3).

#### 4. DISCUSSION

Within two-queen colonies, the queen losing less mass had significantly more worker offspring than her nestmate. A similar pattern was found across colonies: the lower the investment (as measured by loss of body mass) provided by the queen losing less mass, the greater her achieved relative maternity. Because differential mass loss was also associated with survival (as previously found; Balas & Adams 1996; Bernasconi & Keller 1996) there was an association between survival and maternity. In two-queen colonies the queen most likely to survive was the one with more offspring.

In a similar study Balas & Adams (1996) failed to find any significant association between differential maternity and queen survival in two-queen colonies. However, the low polymorphism of mitochondrial markers used in their study allowed assignment of maternity in only 14 out of 48 colonies. Due to this limited sample size, statistical power was presumably low.

Our finding of a negative association between relative mass loss and maternity of nestmate queens is in apparent contradiction with Balas & Adams (1996) finding of a positive association between absolute queen mass loss and brood production. However, the questions addressed differ. We compared relative mass loss and relative maternity of queens within colonies, whereas Balas & Adams (1996) compared absolute mass loss and absolute maternity of the 28 queens from the 14 colonies. Hence our analysis specifically tested whether differential investment of queens within colonies influences relative maternity. By contrast, Balas & Adams (1996) analysis considered simultaneously the effects of within and between colony difference in queen investment, and scored a variable sample of the brood (all adults and pupae), while we scored a roughly constant number of offspring in each colony. Since the total number of worker brood produced during colony-founding is positively correlated with total mass loss of queens in two-queen colonies (Tschinkel 1993; Balas & Adams 1996; Bernasconi & Keller 1996), and there is variation between colonies in total mass loss, it is not surprising that our analyses gave different results.

Table 3. Mean proportion ( $\pm s.d.$ ) of the combined mass loss and of the offspring due to the queen losing less mass in two-queen colonies and in the control ( $U_{\min}$  = comparison with Mann-Whitney  $U$ -test;  $z$  = comparison with  $z$ -test after Fisher  $z$ -transformation)

	two-queen colonies	control (solitary queens)	
proportion of mass loss ( $p_M$ )	$0.44 \pm 0.05$	$0.44 \pm 0.02$	$U_{\min} = 236$ $p = 0.22$
proportion of offspring ( $p_O$ )	$0.58 \pm 0.18$	$0.31 \pm 0.18$	$U_{\min} = 77$ $p < 0.001$
correlation ( $r_s$ ) between $p_M$ and $p_O$	$-0.52$	$0.65$	$z = -4.5$ $p < 0.002$
across colonies	(figure 1a)	(figure 1b)	
number of cases	27 colonies	22 paired values	

The differences in investment roles of queens revealed by our study cannot simply be ascribed to intrinsic differences in their condition. First, we paired queens of equal initial mass, mass being a likely indicator of condition in founding queens (Keller & Passera 1989). Second, the relationship between loss of body mass and maternity was of opposite sign when calculated for control pairs of solitary queens. That is, the relative number of worker offspring produced by solitary queens of the same initial mass was positively correlated with their relative loss of body mass. Thus, the negative relationship in two-queen colonies between energy invested by individual queens and their realized maternity must stem from interactions between queens.

Together these data indicate that there is a division of labour between queens during cooperative colony founding, with one of the two queens investing more energy and being more likely to die, whereas the other queen achieves higher maternity and is more likely to survive. It is, as yet, unclear how queens settle on these different roles. Queens extensively antennate each other's head and gaster, and remain lined up in close body contact for long time periods after being joined in one nest. During most of early colony founding, they typically maintain contact by continuously wagging their antennae towards each other's body and extremities and the common brood pile (G. Bernasconi, personal observation). This behaviour may allow mutual assessment of potential asymmetries in fighting ability and lead to differential reproductive and investment roles among nestmate queens.

The different roles of nestmate queens were temporally stable. The apportionment of maternity for early and late offspring (adult workers and worker larvae) were significantly correlated. Similarly, the relative mass loss of nestmate queens was previously found to remain stable over time (Bernasconi & Keller 1996). The persistent difference between cofoundresses in loss of body mass and realized maternity may result either from unequal partitioning of costly tasks such as brood care, and/or differential oophagy. Fire ant foundresses produce both embryonated and trophic eggs, which are fed to larvae (Voss & Blum 1987). If differential production of embryonated and trophic eggs occurs, it may account for the lower maternity and higher body mass loss of the queen producing more trophic eggs. Alternatively, if differential oophagy occurs, it may result in reduced mass loss of the queen eating more eggs, and possibly in higher egg-laying rates, if the eggs eaten are metabolized to produce new eggs. In particular, differential oophagy could affect the apportionment of realized maternity between queens, because the nutritional state of founding fire ant queens is likely to be negatively correlated with the production of trophic, instead of embryonated, eggs (Voss & Blum 1987). Thus, to explain our findings by differential oophagy it is not necessary that nestmate queens recognize their eggs. Indeed, a study of multiple-queen association of the ant *Leptothorax acervorum*, showed that queens fail to do so (Bourke 1994). Strong circumstantial evidence indicates that oophagy occurs when fire ant queens are in association (Tschinkel 1993) but not when alone (Voss & Blum 1987). It remains to be investigated whether fire ant queens differ in the rate of oophagy.

The finding that the queen most likely to win fights is the one with above-average maternity may explain why workers seem not to interfere with queen fights, but apparently direct their attacks against already-injured queens (Balas & Adams 1996; Bernasconi & Keller 1996). Because the surviving queen in most colonies was the mother of the more numerous offspring, the workers may do best by letting the queens fight as an effective rule of thumb to increase the survival prospects of their mother. This may be particularly true if workers cannot reliably discriminate between their mother and other nestmate queens. Theoretical studies indicate that within-colony recognition mediated by genetic cues might be unstable and error prone (see Keller 1997). First, genetic models show that allelic diversity of recognition systems should decrease over time because more frequent alleles will be continually favoured until fixation (Crozier 1988; Ratnieks 1991). Second, the use of genetically specified cues is associated with relatively high rates of recognition errors since recombination results in different combinations of segregating alleles in family members (Lacy & Sherman 1983; Waldman 1987). Finally, workers may also benefit by muting or scrambling recognition labels in social insect colonies when nepotism entails costs for colony productivity that outweigh the benefits of nepotism (Reeve 1997). Label scrambling hampers recognition of sibships within colonies and thus reduces the potential for actual conflicts. The same effect has been proposed to explain the positive correlation between chromosome number and the incidence of eusociality in insects (Sherman 1979; Seger 1983), with high chromosome number decreasing the variance in inclusive fitness (Templeton 1979).

Consistent with the conclusion that within-colony kin recognition based on genetic cues is unstable, there is currently no undisputed evidence of nepotism occurring among matriline or patriline within social insect colonies (Keller 1997). In particular, experimental manipulation of relative maternity composition of the brood did not significantly affect survival of fire ant cofoundresses, indicating that workers are apparently unable to recognize and favour their mother on the basis of genetic cues (Balas & Adams 1996; Bernasconi & Keller 1996). Experiments in mature colonies also indicate that fire ant workers fail to recognize their mother from unrelated queens (DeHeer & Ross 1997). Thus, if workers cannot reliably recognize their mother (Reeve 1997) and/or nepotistic behaviour by workers entails important costs for the colony (Cole 1983; Reeve 1997), the most efficient means by which fire ant workers may maximize their inclusive fitness during colony founding may be by letting the queens fight until death.

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