Ant workers selfishly bias sex ratios by manipulating female development

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Kin selection theory predicts that social insects should perform selfish manipulations as a function of colony genetic structure. We describe a novel mechanism by which this occurs. First, we use microsatellite analyses to show that, in a population of the ant *Leptothorax acervorum*, workers' relatedness asymmetry (ratio of relatedness to females and relatedness to males) is significantly higher in monogynous (single-queen) colonies than in polygynous (multiple-queen) colonies. Workers rear mainly queens in monogynous colonies and males in polygynous colonies. Therefore, split sex ratios in this population are correlated with workers' relatedness asymmetry. Together with significant female bias in the population numerical and investment sex ratios, this finding strongly supports kin-selection theory. Second, by determining the primary sex ratio using microsatellite markers to sex eggs, we show that the ratio of male to female eggs is the same in both monogynous and polygynous colonies and equals the overall ratio of haploids (males) to diploids (queens and workers) among adults. In contrast to workers of species with selective destruction of male brood, *L. acervorum* workers therefore rear eggs randomly with respect to sex and must achieve their favoured sex ratios by selectively biasing the final caste (queen or worker) of developing females.

Keywords: caste; Formicidae; kin selection; sex allocation; social insect

1. INTRODUCTION

Hamilton's (1964) theory of kin selection successfully predicts not just altruism between relatives but also the occurrence of selfish behaviour (Trivers & Hare 1976), even within colonies of highly related social insects (Seger 1991; Bourke & Franks 1995; Crozier & Pamilo 1996; Keller 1999). However, little is known about the mechanisms involved in many kinds of selfish behaviours (Queller & Strassmann 1998; Chapuisat & Keller 1999). Moreover, the most compelling evidence for kin-selected selfishness is found when colony members achieve predicted effects by performing manipulations of brood that are conditional on their colony's relatedness structure (Queller & Strassmann 1998; Chapuisat & Keller 1999). Demonstrations of the mechanisms underlying such facultative, selfish manipulations are very rare and to date both known instances involve selective destruction of male brood (Sundström *et al.* 1996; Foster & Ratnieks 2000).

In the social Hymenoptera, females are diploid and males are haploid and female caste (development as queen or worker) depends in almost all cases on the rearing environment (Wheeler 1986). Split sex-ratio theory (Boomsma & Grafen 1990, 1991) states that, where workers' relative relatedness to new queens and males (relatedness asymmetry) varies within populations, workers maximize their inclusive fitness by rearing mainly queens in colonies with comparatively high relatedness asymmetry and mainly males in colonies with comparatively low relatedness asymmetry. Therefore, in populations with partial multiple mating, workers should mainly raise queens in colonies headed by a singly mated queen and males in colonies headed by a multiply mated queen. Likewise, in populations in which the queen number varies, workers should concentrate on raising queens in single-queen (monogynous) colonies and males in multiple-queen (polygynous) colonies (Boomsma & Grafen 1990, 1991). In principle, workers can bias colony sex ratios by selectively destroying male brood or by altering the fraction of diploid brood that they rear as queens (Pamilo 1991). Sundström *et al.* (1996) have shown that in a monogynous population of the ant *Formica exsecta* in which queens vary in mating frequency, sex ratios are split as predicted and that, in colonies with a singly mated queen, workers bias sex allocation by selectively killing developing males.

In some populations of the facultatively polygynous ant *Leptothorax acervorum*, sex ratios are also split, with monogynous colonies producing mainly females and polygynous colonies producing mainly males (Chan & Bourke 1994; Chan *et al.* 1999; Heinze *et al.* 2001). In this paper, we demonstrate that queen number covaries with workers' relatedness asymmetry, and hence that sex ratios are split in this species as the theory proposed by Boomsma & Grafen (1990, 1991) predicts. We also determine the sex ratio among eggs in monogynous and polygynous colonies and compare this primary sex ratio with the ratio of haploids to diploids among adults and the numerical sex ratio in the two colony types, with these last two ratios being calculated from existing data (Chan *et al.* 1999). We find that *L. acervorum* workers do not selectively kill developing males but instead facultatively manipulate female brood into development as queens or workers in order to achieve their predicted sex-ratio optima. This behaviour occurs conditionally upon the relatedness structure of the colony and is selfish because it leads to female-biased sex allocation contrary to the interests of queens. We therefore demonstrate a novel mechanism whereby ant workers facultatively achieve their preferred, kin-selected sex ratios.

2. MATERIAL AND METHODS

(a) *Determination of relatedness asymmetry and the primary (egg) sex ratio*

Leptothorax acervorum was studied in a facultatively polygynous population in Santon Warren, Thetford Forest, Norfolk, UK. In this population, polygynous colonies make up 20–50% of all colonies and contain, on average, three to five related queens, almost all of which are singly mated (Chan & Bourke 1994; Heinze *et al.* 1995a; Chan *et al.* 1999; Hammond *et al.* 2001). To determine workers' and queens' relatedness asymmetry and the primary sex ratio, we typed individuals at one to five microsatellite loci in 20 colonies collected in 1999 from the Santon Warren population. These 20 colonies (SD 99 sample) were taken from a larger sample collected on 3 June and 10 June 1999 as described by Hammond *et al.* (2001). (They comprise the subset of 20 SD 99 colonies selected for 'detailed genetic analysis' by Hammond *et al.* (2001).) Whole colonies were extracted from their nests (decaying twigs) within 2–3 days of collection and immediately frozen at −70 °C. Therefore their relatedness structure and composition reflected that of colonies in the field. The 20 selected colonies were sexually productive and, in the case of polygynous colonies, contained between two and eight queens, which is the range of queen number shared by 75% of polygynous colonies in the population (Chan *et al.* 1999). Eleven colonies were monogynous (including one queenless colony whose genetic structure revealed that it had recently lost its single queen: Hammond *et al.* 2001) and nine were polygynous.

The five microsatellite loci used were LXAGT1, LXAGA1, LXAGA2 (Bourke *et al.* 1997), MYRT3 (Evans 1993) and L18 (Foitzik *et al.* 1997). In the study population, these loci have beween 5 and 40 alleles and expected heterozygosities between 0.63 and 0.96. Molecular methods were as described by Hammond *et al.* (2001). We measured workers' relatedness asymmetry directly as the ratio of relatedness of existing, adult workers ('old' workers, OW) with female ('new' queens, NQ) and male (M) sexual brood (cf. Brown & Keller 2000). We also measured the relatedness asymmetry of dealate queens (DQ, queens heading colonies). For this purpose, a total of 550 individuals were genotyped from seven monogynous and five polygynous colonies producing both sexes in the SD 99 sample (mean ± s.d. of individuals typed per colony: DQ, 1.9 ± 1.4; OW, 14.9 ± 0.3; NQ, 13.3 ± 5.6; M, 15.7 ± 6.9) at all five microsatellite loci. Regression relatedness was calculated from the genotypic data with the program RELATEDNESS 5.07, which uses algorithms developed by Queller & Goodnight (1989). Relatedness asymmetry was defined as the 'life-for-life' relatedness of queens or workers with new queens divided by 'life-for-life' relatedness of queens or workers with males (Boomsma & Grafen 1990). We converted regression relatednesses to 'life-for-life' relatednesses by multiplying regression relatedness with males by 0.5 (Boomsma & Grafen 1991).

The primary sex ratio (egg haploid : diploid ratio, EHDR, which we express throughout as the proportion of all eggs that are haploid) was estimated by genotyping a total of 821 eggs from the 11 monogynous (mean per colony = 40.6 eggs, range = 21–63 eggs) and nine polygynous (mean per colony = 41.6 eggs, range = 30–63 eggs) SD 99 colonies. Egg genotypes came from up to four of the five loci (all except MYRT3). Male eggs were defined as those appearing homozygous at four loci and female eggs as those with at least one heterozygous locus (cf. Ratnieks & Keller 1998; Passera *et al.*

2001). The probability of misclassifying female eggs as male was negligible, as none of 713 adult or pupal females typed by Hammond *et al.* (2001) was homozygous at more than two of these loci. Diploid males (Cook & Crozier 1995) were very rare (Hammond *et al.* 2001) and almost all male eggs were laid by queens (R. L. Hammond *et al.*, unpublished data).

(b) *Analysis of numerical sex ratio and haploid : diploid ratio among adults from 1993–1995 samples*

Previous work has shown that in the Santon Warren population of *L. acervorum*, monogynous colonies produced a female-biased sex investment ratio and polygynous colonies produced a male-biased sex investment ratio consistently over 3 years of study (1993–1995: Chan & Bourke 1994; Chan *et al.* 1999). We compared the primary sex ratio determined from the SD 99 colonies with adult production calculated from the 1993–1995 data to investigate whether the proportion of haploid eggs laid by queens differs from the proportion of haploid adults reared by workers in the population. This comparison makes two assumptions. First, it assumes that the primary sex ratio among eggs present in nests in June is representative, in both types of colony, of that among the cohort of eggs yielding adult queens, males and workers. Second, it assumes that patterns of resource allocation, including sex allocation, remain similar across years in the study population. Evidence that this assumption is justified comes from the fact that the pattern of sex-ratio splitting remained constant over the three previous years investigated (Chan *et al.* 1999), as did the pattern of haploid : diploid resource allocation among adults (see below). In addition, in the 25 colonies of the entire SD 99 sample producing 10 or more adult sexuals, the mean numerical sex ratios for polygynous colonies ($n = 9$) and monogynous colonies ($n = 16$) were 59% and 32% adult males respectively. Neither of these values was significantly different from the respective average sex ratio for each colony class in the 1993–1995 data (see below; general linear model (GLM), $F_{3,133} = 1.70$, $p > 0.05$), demonstrating that the pattern of sex-ratio splitting occurring in 1999 was the same as that found in 1993–1995. (These data were not used in the overall analysis because worker production was not measured in the SD 99 colonies. The adult sex-ratio data from the SD 99 colonies were not compared to the primary sex-ratio data in paired comparisons because they stemmed from different cohorts of brood, since *L. acervorum* sexuals take at least a year to develop to adulthood (Buschinger 1973).)

We therefore reanalysed the 1993–1995 data to calculate the numerical sex ratio among adults (NSR, number of adult males as a proportion of all sexual production, i.e. males and new queens) and the adult haploid : diploid ratio (AHDR, number of adult males as a proportion of all production, i.e. males, new queens and new workers). NSR and AHDR were calculated from data on 86 monogynous and 30 polygynous colonies collected from Santon Warren in 1993–1995 and published by Chan & Bourke (1994) and Chan *et al.* (1999). To reduce errors in estimating proportions, we included in this dataset only colonies producing 10 or more new workers or sexuals. Analysis of patterns of variation among NSR, AHDR and EHDR were carried out using GLMs. Overall NSR for the entire population of monogynous and polygynous colonies was calculated within and across years from the 1993–1995 data (Chan *et al.* 1999) and the 1999 data. For this analysis, means and confidence intervals were calculated by weighting colonies by their average sexual productivity using J. J. Boomsma's method, which is described

by Bourke & Franks (1995, p.160). All datasets were tested for normality prior to analysis and none proved significantly non-normal (Kolmogorov–Smirnov tests, range of p values = 0.06–0.97).

3. RESULTS

(a) Workers' and queens' relatedness asymmetry

In monogynous colonies, the overall (± s.e.) worker-to-sexual 'life-for-life' relatedness values were $R_{OW–NQ}$ = 0.68 ± 0.04 and $R_{OW–M}$ = 0.21 ± 0.06 (n = 7 colonies from the SD 99 sample). In polygynous colonies, they were $R_{OW–NQ}$ = 0.23 ± 0.04 and $R_{OW–M}$ = 0.12 ± 0.04 (n = 5 colonies from the SD 99 sample). Workers' relatedness asymmetry was significantly higher in monogynous colonies (median 3.4) than in polygynous colonies (median 2.0) (Mann–Whitney test, W = 59.0, n = 12 colonies, $p < 0.05$). By contrast, dealate queens were related to female and male sexuals in the monogynous colonies by $R_{DQ–NQ}$ = 0.40 ± 0.03 and $R_{DQ–M}$ = 0.50 ± 0.01 (n = 6 colonies, since one 'monogynous' colony was queenless; see § 2) and in the five polygynous colonies by $R_{DQ–NQ}$ = 0.12 ± 0.07 and $R_{DQ–M}$ = 0.14 ± 0.03. Queens' relatedness asymmetry in monogynous colonies (median 0.8) was not significantly different from that in polygynous colonies (median 0.6) (W = 39.0, n = 11 colonies, $p > 0.65$).

These results were consistent with monogynous colonies consisting mainly of colonies headed by one, singly mated queen and with polygynous colonies, on average, containing several, related, singly mated queens (Hammond *et al.* 2001). They were also consistent with previous inferences regarding the genetic structures of monogynous and polygynous colonies, made on the basis of differences in relatedness among workers (Chan & Bourke 1994; Heinze *et al.* 1995*a*; Chan *et al.* 1999; Hammond *et al.* 2001).

(b) Comparison of primary sex ratio with numerical sex ratio and haploid : diploid ratio among adults

The numerical sex ratio among adults (mean proportion of males ± 95% confidence interval) for the whole population across all years (1993–1995, 1999) was 0.40 ± 0.04 (n = 141 colonies), i.e. significantly female-biased. This confirmed the finding reported by Chan *et al.* (1999) of significant female bias in the overall sex investment ratio, since in *L. acervorum* numerical and investment ratios differ only slightly because of a low degree of sexual size dimorphism (Chan *et al.* 1999). The yearly means of NSR (± 95% confidence interval) were 0.50 ± 0.09 in 1993, 0.43 ± 0.10 in 1994, 0.31 ± 0.06 in 1995 and 0.34 ± 0.10 in 1999. Variation across years was significant (ANOVA, $F_{3,137}$ = 4.12, $p < 0.01$), but data were pooled across years because the across-year mean represents the best estimate for the average year. The population sex investment ratio for 1993–1995 did not vary significantly across years (Chan *et al.* 1999).

Within colony types there were no significant effects of year on NSR (GLM, $F_{2,110}$ = 3.01, $p > 0.05$) or AHDR (GLM, $F_{2,110}$ = 2.81, $p > 0.05$), and no significant interactions of year with colony type, so overall NSR and AHDR for each colony type were calculated from data pooled across years. Consistent with the results of Chan *et al.*
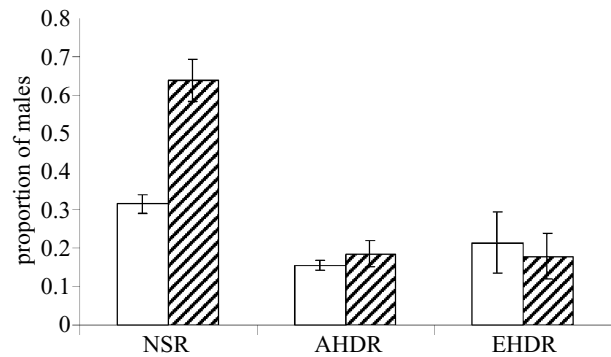

Figure 1. Mean proportion (± 1 s.e.) of males in monogynous (white bars) and polygynous (hatched bars) *L. acervorum* colonies. Abbreviations: NSR, number of adult males as a proportion of all sexual production—data from 86 monogynous and 30 polygynous colonies collected between 1993 and 1995 from Santon Warren, UK (Chan & Bourke 1994; Chan *et al.* 1999); AHDR, number of adult males as a proportion of all production—data as for NSR; EHDR, number of male eggs as a proportion of all eggs—data from 11 monogynous and 9 polygynous colonies collected in 1999 from Santon Warren, UK.

(1999) for the sex investment ratio, polygynous colonies were found to produce a significantly higher mean proportion of adult males (64%) than monogynous colonies (32%) (GLM, $F_{1,110}$ = 22.3, $p < 0.001$; figure 1, NSR). However, the overall ratio of males to females (queens plus workers) among adults did not vary across the colony types (GLM, $F_{1,110}$ = 0.29, $p > 0.05$; figure 1, AHDR). Hence, polygynous colonies reared a significantly smaller mean fraction of all brood as sexuals (queens or males) compared with monogynous colonies (26% vs 48%, GLM, $F_{1,110}$ = 36.0, $p < 0.001$). Consistent with this, polygynous colonies had significantly larger workforces than monogynous colonies (Chan *et al.* 1999). However, neither the mean total production per colony (dry weight of new queens, males and new workers) nor the mean total productivity per worker differed significantly between colony types (Chan *et al.* 1999).

Comparison of the frequency of haploids among eggs (the primary sex ratio, EHDR) and adults (sexuals plus workers, AHDR) showed that the overall mean fraction of haploids was 16% (figure 1, AHDR versus EHDR), with no significant effect of either age (egg vs adult) or colony type (monogynous vs polygynous) and no significant interactions between these factors (GLM, all $F_{1, 132} < 0.886$, all $p > 0.348$). This demonstrates that, in both monogynous and polygynous colonies, queens lay the same fraction of haploid eggs and workers rear eggs to adulthood randomly with respect to the ploidy of brood.

4. DISCUSSION

Two main conclusions are drawn from this study. We found that workers' but not queens' relatedness asymmetry was significantly higher in monogynous than in polygynous colonies. Monogynous colonies also produce significantly more female-biased sex investment ratios than polygynous colonies (Chan & Bourke 1994; Chan *et al.* 1999). Therefore, workers' relatedness asymmetry is associated with sex allocation in the study population in
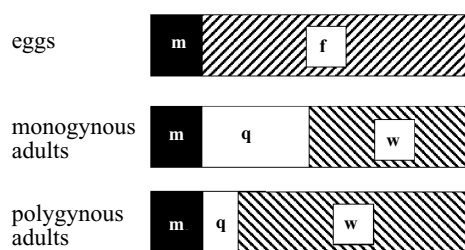
Figure 2. Diagram of resource allocation during the development of brood from egg to adult in *L. acervorum* colonies; the width of bars represents the proportions of sexes and castes reared. Eggs consist of 16% male (m) eggs and 84% female (f) eggs. The fraction of males remains the same among adults, so workers therefore generate a split sex ratio by rearing a higher fraction of females as queens (q) in monogynous colonies compared with polygynous colonies, which rear a higher fraction of females as workers (w).

the direction predicted by Boomsma & Grafen's (1990, 1991) split sex-ratio theory. This finding confirms the conclusions of Chan & Bourke (1994) and Chan *et al.* (1999) and adds to existing support for the theory of Boomsma & Grafen (Queller & Strassmann 1998). In another species of facultatively polygynous ant, *Myrmica tahoensis*, and in a population of *L. acervorum* in Germany, sex ratio has been found to covary with inferred workers' relatedness asymmetry as the theory predicts, but this relatedness asymmetry was not correlated with queen number (Evans 1995; Heinze *et al.* 1995*b*, 2001). This difference could arise if differing patterns of queen turnover (Heinze *et al.* 1995*b*; Bourke *et al.* 1997) between populations lead in some cases to a coupling of workers' relatedness asymmetry with queen number and in other cases to a decoupling of these traits (Chan *et al.* 1999; Heinze *et al.* 2001). In addition, the occurrence of split sex ratios primarily associated with workers' relatedness asymmetry (and, in some populations, incidentally linked to queen number) supports the view that workers in facultatively polygynous ant populations assess their relative relatedness asymmetry not from queen number itself, but from genetic odour diversity in the brood (Evans 1995). In a further species, sex ratios covaried with queen number but not workers' relatedness asymmetry, suggesting that in some polygynous ants other factors also generate split sex ratios (Brown & Keller 2000).

Second, our findings allow us to infer a mechanism by which workers achieve split sex ratios in the Santon Warren population of *L. acervorum*. We found that the primary sex ratio was the same (16% haploids) in both monogynous and polygynous colonies and that it also equalled the frequency of haploids among adults, whereas the numerical sex ratio and the proportion of sexuals raised differed significantly across colony types. Workers must therefore achieve their favoured sex ratios not by selectively destroying males in monogynous colonies but by rearing relatively more female eggs as adult queens in monogynous colonies than in polygynous colonies (figure 2).

The observed difference in resource allocation to queens or workers between the colony types (figure 2) also reflects expected life-history differences between them (Bourke & Heinze 1994; Chan & Bourke 1994), with polygynous colonies predicted to produce relatively more

workers because of reproduction by colony budding (Pamilo 1991). However, neither this factor nor queen–worker conflict over queen replacement (Bourke & Chan 1999) can explain all the additional females reared as queens in the population. This follows from our finding that the overall numerical and investment sex ratios (average NSR = 40% males) of the population are significantly female-biased, as predicted if population-level sex allocation is determined by the workers' kin-selected interests (Chan *et al.* 1999). Such a sex ratio is contrary to the interests of queens, which, in the absence of local mate competition in the study population (Chan *et al.* 1999; Hammond *et al.* 2001), should favour an unbiased or a male-biased population sex ratio (Trivers & Hare 1976; Pamilo 1990). In short, the female-biased population sex ratio and split sex ratios that correlate with workers' relatedness asymmetry are consistent with worker-controlled sex allocation under kin selection at both population and colony level. Our findings therefore represent the first demonstration of facultative worker biasing of female caste fate (Bourke & Ratnieks 1999) as a mechanism of adaptive sex-ratio manipulation. Furthermore, by demonstrating selfish behaviour occurring conditionally on colony relatedness structure, they provide strong support for kin-selection theory.

This conclusion raises the question of why *L. acervorum* should display a means of facultative sex allocation not involving selective male destruction as in *F. exsecta* (Sundström *et al.* 1996) or involving queens exhibiting different primary sex ratios in different colony classes as has been found in *F. exsecta* (Sundström *et al.* 1996), *Linepithema humile* (Keller *et al.* 1996*b*) and *Solenopsis invicta* (Aron *et al.* 1995; Passera *et al.* 2001). In *F. exsecta*, sexuals are reared from cohorts of eggs that are largely separated, in time, from eggs yielding workers (Sundström *et al.* 1996). By contrast, in *L. acervorum* there appears to be one, extended period of egg-laying each year and new workers eclose at the same time as new sexuals (A. F. G. Bourke and R. L. Hammond, unpublished observations). We suggest that simultaneous sexual-rearing and worker-rearing in *L. acervorum* provides workers with an opportunity to manipulate sex allocation via adjusting the caste fate of developing females that is not available to workers of species with separate sexual and worker cohorts.

Another possible reason for the difference between the methods of sex-ratio control in *F. exsecta* and *L. acervorum* is that workers of the latter species might be incapable of discriminating haploid from diploid eggs or larvae (cf. Nonacs & Carlin 1990; Chapuisat *et al.* 1997). However, if *L. acervorum* workers cannot discriminate haploid from diploid brood, a further question is raised: why do *L. acervorum* queens in monogynous colonies not lay relatively more male eggs (given queens in all colonies should favour less female-biased sex allocation than workers)? *Leptothorax acervorum*, with no difference between the primary sex ratios in monogynous and polygynous colonies, resembles species in which queens have been found to produce the same primary sex ratio in different classes of colony (Keller *et al.* 1996*a*; Aron *et al.* 1999) but contrasts with other species in which primary sex ratios do differ between colony classes (see above). In *S. invicta*, queens in some colonies produce a relatively male-biased primary sex ratio, and it has been proposed that the corresponding

relative lack of diploid eggs accounts for the high level of adult male production observed in these colonies (Passera *et al.* 2001). *Leptothorax acervorum* queens from both colony types in the study population lay many more eggs than workers rear to adulthood (Bourke 1993; R. L. Hammond *et al.*, unpublished data). This suggests that producing a higher frequency of male eggs in monogynous colonies would be an ineffective means of regaining sex-ratio control for *L. acervorum* queens, because workers could circumvent it via their probable control of the sexualization of the relatively plentiful supply of developing diploid brood. In addition, an excess of eggs means that workers could bias female brood towards development as queens or workers, for example by differential feeding of developing larvae (Wesson 1940; Wheeler 1986; Buschinger 1990), without necessarily incurring significant costs either in terms of constraining resource allocation to the other caste or in terms of reducing overall colony productivity (cf. Reuter & Keller 2001). In sum, as other studies have shown (e.g. Helms 1999; Passera *et al.* 2001), differences in life-history features between social insect species are likely to be important in determining the scope and nature of the mechanisms by which parties achieve their fitness interests in kin-selected conflicts.

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