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Evolution

Relatedness threshold for the production of female sexuals in colonies of a polygynous ant, *Myrmica tahoensis*, as revealed by microsatellite DNA analysis

(sex ratio/altruism/social insects/kin selection/polygyny)

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ABSTRACT **The genetic relationships of colony members in the ant *Myrmica tahoensis* were determined on the basis of highly polymorphic microsatellite DNA loci. These analyses show that colonies fall into one of two classes. In roughly half of the sampled colonies, workers and female offspring appear to be full sisters. The remaining colonies contain offspring produced by two or more queens. Colonies that produce female sexuals are always composed of highly related females, while colonies that produce males often show low levels of nestmate relatedness. These results support theoretical predictions that workers should skew sex allocation in response to relatedness asymmetries found within colonies. The existence of a relatedness threshold below which female sexuals are not produced suggests a possible mechanism for worker perception of relatedness. Two results indicate that workers use genetic cues, not queen number, in making sex-allocation decisions. (*i*) The number of queens in a colony was not significantly correlated with either the level of relatedness asymmetry or the sex ratio. (*ii*) Sex-ratio shifts consistent with a genetically based mechanism of relatedness assessment were seen in an experiment involving transfers of larvae among unrelated nests. Thus workers appear to make sex-allocation decisions on the basis of larval cues and appear to be able to adjust sex ratios long after egg laying.**

Workers in eusocial colonies of ants, bees, and wasps (order Hymenoptera) are unequally related to the male and female reproductives they provision. This relatedness asymmetry results from the haplodiploid genetic system common to all Hymenoptera (1). Because males are haploid, full sisters are related by $r = 3/4$, while females are related to their brothers by only $r = 1/4$ and to their sons (for species in which workers lay haploid male eggs) by $r = 1/2$ (1, 2), where r is the "life-for-life" coefficient of relatedness (2). Accordingly, sex investment ratios are predicted to be highly female biased in hymenopteran species where workers control sex allocation, when colonies are headed by single once-mated queens (1). The asymmetry and consequent female bias should be less extreme in species where colonies have several related queens or single queens that mated more than once (1, 3). These two predictions are broadly supported by interspecific comparisons, but sex-ratio differences among species could be caused by factors other than relatedness asymmetry, including competition for mates among related males (4), competition for resources among females (5), and other ecological (6) or phylogenetic correlates of colony structure.

When relatedness asymmetries vary from colony to colony within a population, workers can increase their inclusive fitness (2) by specializing on the sex to which they are relatively more closely related (1, 7, 8). Bimodal sex-ratio distributions (where some colonies produce mostly males and others produce females) are common in ants, providing a basis for powerful tests of the responses of hymenopteran workers to relatedness asymmetries (7–9). Protein electrophoretic data from colonies of a monogynous (single queen) wood ant, *Formica truncorum*, were recently used to show that colonies specialize on males or females in accordance with the relative relatedness asymmetry of their workers (9). *F. truncorum* workers appear to use genetic cues to assess the number of patrilines in their colony, producing less female-biased sex ratios, on average, when this number exceeds one.

Here I present genetic evidence that split sex ratios in a polygynous ant reflect colony-level variation in relatedness. In the facultatively polygynous ant *Myrmica tahoensis*, queen number is the main cause of variation in relatedness asymmetries because queens generally mate only once (10). Thus workers in this species might use either queen number or a more direct estimate of relatedness in making sex allocation decisions. To distinguish between these alternatives, I censused queens and sexuals in reproductive colonies and estimated relatedness within each colony by using genotypes at three highly polymorphic microsatellite loci (10, 11).* These relatedness estimates suggest that *M. tahoensis* workers assay genetic relatedness directly when making sex-ratio decisions. An apparent relatedness threshold, below which female sexuals are not produced, provides insight into the mechanisms used for assessing relatedness. I also describe a field manipulation of larval relatedness. The results of this experiment imply that workers skew the sex ratio late in larval development, in response to their perception of cues presented by larvae.

MATERIALS AND METHODS

Specimen Collection. Field colonies of *M. tahoensis* were marked and censused during the summers of 1990–1993, at three sites near the Rocky Mountain Biological Laboratory (RMBL) in Colorado. Complete colonies were excavated in late July and early August of 1992 and 1993, and sexual offspring, workers, and queens were removed and sorted for counting and genetic analyses. Colonies were dug near midday, when most colony members were near the surface. They were placed into plastic bags and then were sorted on large plastic tarps within squares separated by barriers of Tree Tanglefoot (Tanglefoot, Grand Rapids, MI). All colony members were counted and then stored in 95% ethanol in separate microcentrifuge tubes. Investment was calculated on the basis of dry weights from a subset of male and female sexuals. The ants used in this study have been tentatively identified as *M. tahoenis*. Voucher specimens have been deposited at the

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Abbreviation: RMBL, Rocky Mountain Biological Laboratory.
*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L14688–L14690).

Museum of Comparative Zoology (Harvard University, Cambridge, MA) and in the collection of A. Francoeur (University of Quebec, Chicoutimi).

DNA Amplification and Genetic Analyses. DNA was extracted from 10 workers and 10 sexuals (when available) from each of 39 colonies and then genotyped at microsatellite loci *Myrt2, Myrt3*, and *Myrt4* (10). Dried ants were pulverized over dry ice in 0.65-ml microcentrifuge tubes with a hand drill by using a melted pipette tip as a pestle. They were suspended in 200 μl of a lysis buffer (0.05% SDS/0.1 M NaCl/0.2 M sucrose/0.1 M Tris·HCl, pH 9/0.05 M EDTA) and then were incubated for 30 min in a 65°C bath. Impurities were removed by using potassium acetate (28 μl of an 8 M solution), followed by phenol/chloroform extraction. DNA was precipitated by adding 2 vol of absolute ethanol. After a 10-min centrifugation (relative centrifugal force = 12,000 × *g*), pellets were rinsed with 70% ethanol, then dried, and resuspended in 500 μl of TE^{-3} (12).

The 10-μl polymerase chain reaction (PCR) mixtures contained 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 125 μM dATP, 125 μM dCTP, 125 μM dGTP, 125 μM dTTP, 0.5 μM of each primer, 20 nM leading primer end-labeled with [γ-^{32}P]ATP, 0.5 unit of *Taq* polymerase, and 2 μl of DNA extract. Primers for *Myrt2, Myrt3*, and *Myrt4* are as described in ref. 10. Reactions were processed in 96-well trays, by using a Techne Laboratories (Princeton) thermal cycler. Locus *Myrt2* was amplified by using a program of 94°C for 1 min and 45°C for 2 min for 30 cycles. *Myrt3* was amplified with 94°C for 1 min and 55°C for 2 min for 25 cycles. *Myrt4* was amplified with 25 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. PCR products were diluted with 10 μl of formamidë loading dye (12), denatured by heating, and separated electrophoretically on 7% denaturing polyacrylamide gels (10).

Loci *Myrt2, Myrt3*, and *Myrt4* showed 28, 36, and 45 alleles, respectively, in *M. tahoensis*. Rare alleles of similar size were combined, resulting in a total of 25 recognized alleles per locus. Regression estimates based on shared alleles (13) were used to characterize the average relatedness among colony members. These estimates (along with corresponding jackknife error estimates) were made by using the computer program RELATEDNESS 4.2, obtained from D. Queller (Rice University, Houston). Worker–worker relatedness was used as a surrogate for worker–gyne relatedness in male-producing colonies, since this estimate is statistically indistinguishable from that of worker–gyne relatedness when gynes are present. Allele frequencies used to calculate relatedness were derived from a sample of 1101 specimens.

Larval Cross-Fostering Experiment. In the genus *Myrmica*, sexual larvae take two seasons to develop. Larvae reach the third instar in their first year and then overwinter before finishing development and eclosing as adults during their second year (14). Overwintered third-instar larvae were transferred among *M. tahoensis* nests in two sites near the RMBL (Somar and Ridge) in early June 1994. Twenty larvae in each of 80 experimental colonies were removed and replaced with 20 conspecific larvae from donor nests at least 15 m distant. In 80 control nests, 20 larvae were removed, handled in the same way, and then returned to their nest of origin. Experimental colonies contained 100–250 overwintered larvae when the transfers were conducted. All colonies were scored for the presence and gender of reproductive offspring from July 25 to August 5. Colonies of *M. tahoensis* congregate at the soil surface beneath rocks during warm days. Colonies were surveyed (and a subset of the sexuals were collected) by overturning these rocks and collecting individuals with an aspirator.

RESULTS

Colonies of *M. tahoensis* showed strongly split sex ratios, with 83 of 111 colonies (75%) producing either all males or all females. Male-specialist colonies also contained diploid worker pupae. Thus it is unlikely that (haploid) males were produced simply because no mated individuals were present in these nests. Nests produced 13.9 males and 5.0 females, on average, with a maximum of 97 and 91, respectively. Sex ratios were not correlated with the total number of sexuals produced (n = 111 colonies; Kendall's τ = 0.064, not significant). Male- and female-producing colonies showed striking differences in relatedness (Fig. 1). Average relatedness among females was significantly higher in colonies that produced gynes (female sexuals) than in those that produced only males [$\bar{r}$ = 0.77 ± 0.03 vs. $\bar{r}$ = 0.52 ± 0.04 (mean ± SEM), respectively; Wilcoxon rank-sum test P = 0.0007]. Relatedness estimates for gyne-producing colonies were uniformly high ($\bar{r} \geq 0.58$), while those for male-producing colonies varied from $\bar{r}$ = 0.14 to $\bar{r}$ = 0.86. In 12 of the 16 colonies that produced female sexuals, workers and gynes showed genotypic arrays at the three loci consistent with their being the offspring of a single once-mated queen (Fig. 2). A total of 6 workers in the remaining 4 colonies had at least one allele that excluded them from being full sisters with their nestmates, but even in these colonies all gynes appeared to be full sisters to each other and to the most common worker sibship.

Pedigrees in male-producing colonies were considerably more complex, as expected from the lower average relatedness of these colonies. Workers appeared to be full sisters in only 6 of the 23 male-producing colonies. In most colonies, worker genotypes suggested that they were the progeny of two or more related queens, consistent with the expectation that workers in these colonies also experience lower levels of relatedness asymmetry (1). Queen number was poorly correlated with sex allocation (Kruskal–Wallis test with queenless, monogynous, and polygynous classes as factors; χ^2 = 2.79; df = 2; not significant). Both single- and multiple-queen colonies produced all-male and all-female broods, implying that worker perception of relatedness, rather than queen number itself, was the cause of the observed sex-allocation patterns.

The larval transfer experiment produced a clear-cut result in the predicted direction, despite an unexpectedly small sample

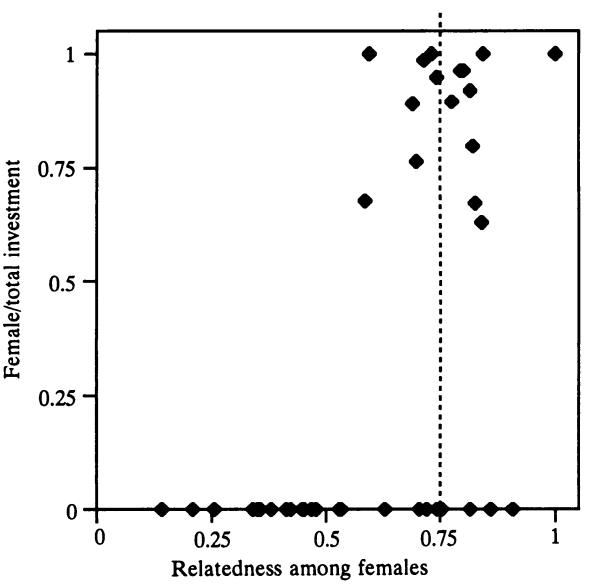

FIG. 1. Proportional investment in gynes as a function of the estimated relatedness of nestmate females. The dotted line indicates the level of relatedness expected in nests with a single once-mated queen.

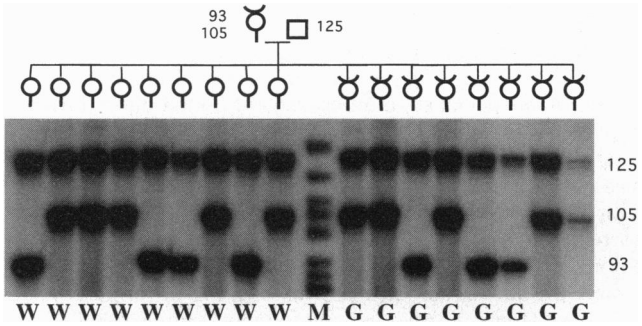

FIG. 2. Autoradiograph showing alleles at microsatellite locus *Myrt4* for female nestmates in colony G12 (W, workers; G, gynes; M, size standards). The predicted pedigree for colony G12, based on genotypes at all three loci used in this study, is shown above the autoradiograph. The genotypes of female offspring from this colony are consistent with their being full sisters, in which case workers are much more closely related to female than to male reproductive offspring [$r_{female} = 0.75$ vs. $r_{male} \leq 0.375$; workers lay many male-destined eggs in this and other species of *Myrmica* (10, 15)]. The genotypes imply that the male parent of these females had allele 125 at locus *Myrt4*, and the queen was a heterozygote for alleles 93 and 105.

size (Table 1). Only 68 of the 160 experimental colonies produced sexuals, a frequency similar to that among unmanipulated nests and probably a consequence of unusually hot dry summer weather. Nonetheless, females were produced by a significantly smaller proportion of experimental nests (those receiving unrelated larvae) than of controls ($G = 7.52$; df = 1; $P = 0.006$).

DISCUSSION

Variation in relatedness is strongly correlated with the sex-ratio variation found among colonies of *M. tahoensis*, in a direction that supports the split sex-ratio theory of Boomsma and Grafen (7, 8). Alternative hypotheses involving (*i*) competition among daughter queens or males (4, 5) and (*ii*) variation in the relative costs of male and female sexuals (3) are not supported because these hypotheses predict associations (which were not found) between colony productivity and sex ratios. Another possible explanation for colony-to-colony sex-ratio variation in this species involves rapid queen turnover. If queens live only 1 or 2 years on average, as suggested by unpublished microsatellite genetic data for this species and allozyme data for the European species *Myrmica ruginodis* and *Myrmica lobicornis* (16), then colony survival will depend on regularly replacing queens when queen numbers are low. Colonies without a queen, or those with only one queen, should be most likely to adopt new queens. Workers in these colonies could increase their inclusive fitness by rearing replacement queens themselves rather than adopting less closely related queens from another colony. Thus the observed association between relatedness and sex allocation might arise as an incidental consequence of the inevitable association between low queen number and high female–female relatedness. But contrary to the prediction of this hypothesis, queen number was poorly correlated with sex allocation. Queenless and single-queen colonies regularly produce all-male sexual broods (75 and 54% of these colonies, respectively), implying that worker perception of relatedness, not queen number, is the cause of the observed sex-allocation patterns.

Table 1. Female sexual production in experimental colonies of *M. tahoensis*

Colonies	Produced female sexuals	Produced only males
Experimental (unrelated larvae introduced), no.	1	34
Control (native larvae reintroduced), no.	8	25
Total, no.	9	59

Proximate mechanisms of kin recognition in social insects are poorly understood. In this study, *M. tahoensis* colonies showed an apparent relatedness threshold, below which only male (not female) reproductives were produced. This suggests that workers may assess relatedness by detecting allelic diversity among larvae in their nests at one or more highly polymorphic loci analogous to major histocompatibility complex loci in mice (17). Ratnieks (18) proposed such a model for monogynous insect colonies, in which workers estimate the number of times their queen has mated by counting the odor phenotypes of their female nestmates. Under this model, workers would shift their allocation toward males when the number of phenotypes exceeded a critical threshold. Ratnieks (18) concluded that error rates would be large, even with considerable allelic diversity at the loci used as cues. More generally, workers could estimate relatedness by separately assessing each allele found within larvae. In this way workers could assess colony genetic diversity more accurately regardless of whether increased diversity resulted from multiple queens or from queens that mated more than once. Further, by scoring each allele independently, workers could accurately identify haploid male larvae as hemizygotes, rather than homozygotes, at relatedness loci. By surveying the 100–250 larvae present in nests each spring, *M. tahoensis* workers could perceive most or all of the genetic cues present in their colony.

The observed correlation between relatedness and sex ratio is consistent with theoretical expectations, but it might nonetheless arise from uncontrolled confounding factors. Direct manipulation of the genetic structures of colonies allows an additional test of worker response to relatedness asymmetries. Mueller (19) removed founding queens from nests of the sweat bee *Augochlorella striata*; the queens were replaced by mated workers, decreasing the expected relatedness asymmetry. As predicted, experimental nests produced more male-biased sexual broods than did unmanipulated controls, but this test did not distinguish between worker perception of genetic relatedness cues and worker perception of queen turnover. The results of the larval-transfer experiment described here imply that *M. tahoensis* workers can make sex-ratio decisions on the basis of cues presented by larvae. This experiment also sheds light on the timing of sex-ratio responses. The point during development at which workers can both assess the sex of larvae and bias the sex ratio is critical for evolutionary models of sex-ratio biasing in Hymenoptera (20). In *M. tahoensis*, colony members apparently detected relatedness cues and then biased their colony's sex allocation in the second season of the 2-year larval development period. This implies that workers can differentially process existing larvae [perhaps by shunting queen-potential larvae into a worker developmental pathway (14)]. Aron *et al.* (21) have shown that brood sex ratios of the ant *Iridomyrmex humilis* (= *Linepithema humile*) change during development, suggesting that workers in many ant species might be able to bias sex ratios secondarily. Since *M. tahoensis* workers lay eggs that develop into males (10), it is possible that workers also influence the initial sex ratio of both eggs and young larvae. Longitudinal surveys of individual colonies will help determine the relative importance of early and late sex-ratio biasing in *M. tahoensis*.

This study provides strong evidence that genetic relatedness, not queen number, affects sex-ratio decisions made by *Myrmica* workers. The relatedness threshold, below which females are not produced, hints at possible mechanisms for the perception of relatedness by workers. Additional experiments involving the manipulation of nestmate relatedness should help to characterize the relatedness-assessment mechanisms

used by *M. tahoensis* and other species in which colony sex ratios vary in response to relatedness asymmetries.

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