Genetic royal cheats in leaf-cutting ant societies

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Social groups are vulnerable to cheating because the reproductive interests of group members are rarely identical. All cooperative systems are therefore predicted to involve a mix of cooperative and cheating genotypes, with the frequency of the latter being constrained by the suppressive abilities of the former. The most significant potential conflict in social insect colonies is over which individuals become reproductive queens rather than sterile workers. This reproductive division of labor is a defining characteristic of eusocial societies, but individual larvae will maximize their fitness by becoming queens whereas their nestmates will generally maximize fitness by forcing larvae to become workers. However, evolutionary constraints are thought to prevent cheating by removing genetic variation in caste propensity. Here, we show that one-fifth of leaf-cutting ant patrilines cheat their nestmates by biasing their larval development toward becoming queens rather than workers. Two distinct mechanisms appear to be involved, one most probably involving a general tendency to become a larger adult and the other relating specifically to the queen–worker developmental switch. Just as evolutionary theory predicts, these "royal" genotypes are rare both in the population and within individual colonies. The rarity of royal cheats is best explained as an evolutionary strategy to avoid suppression by cooperative genotypes, the efficiency of which is frequency-dependent. The results demonstrate that cheating can be widespread in even the most cooperative of societies and illustrate that identical principles govern social evolution in highly diverse systems.

caste | polymorphism | social evolution | polyandry | social insect

Although social groups most obviously exhibit cooperation, they are also often the scene of conflict. Unless group members are clones, their reproductive interests will differ, and individuals may benefit by exploiting the cooperative efforts of other group members (1). Genetic polymorphism for cheating is therefore predicted to arise in all nonclonal social systems (2, 3) and is particularly well characterized in the social microbes (4–6). However, such genetic cheating does not appear to occur in one of the classic examples of cooperation, the development of social insect larvae into either reproductive queens or sterile workers. This lack of genetic cheating is thought to be because queens and workers normally differ morphologically, the caste destiny of larvae is determined by environmental cues, and these cues are controlled by adult workers that are unable to bias larval development nepotistically (7–10). The only known exception to this lack of genetic variation for queen–worker developmental propensity is restricted to artificially induced emergency queen production in honeybees (11–14), queens of which are normally determined solely by being fed a special diet of "royal jelly." However, whether constraints prevent genetic variation for caste destiny under normal conditions, or whether, as predicted by evolutionary theory, it in fact occurs but is rare and thus hard to detect, is unknown.

The leaf-cutting ant *Acromyrmex echinatior* is particularly suitable for examining whether rare, cheating lineages occur because queens mate with multiple (haploid) males (polyandry) (15). Their worker and queen offspring (diploid females) thus consist of a number of full-sister lineages (patrilines) that are half-sisters toward each other. Individuals of different patrilines within colonies share the same rearing conditions and, when colonies are headed by a single mother queen (monogyny), the same maternal genotype on average. They therefore differ only in their paternal genotype, which enables the ready detection of genetic variation. Patrilines of *A. echinatior* are known to differ in their propensities to develop into the two main worker castes [large workers (LW) or small workers (SW)] (16, 17), which may improve the efficiency of colony division of labor (16–20). To establish whether royal patrilines occur, we sampled workers and daughter queens (gynes sensu stricto) from five mature, monogynous colonies of *A. echinatior*. We then genotyped them at four polymorphic microsatellite loci and assigned them to the different patrilines within each colony.

Results and Discussion

Significant differences in patriline representation between queens and workers were found in three colonies (Fig. 1). The result was not due solely to very rare patrilines (Fig. 1) or to false discoveries resulting from multiple tests [supporting information (SI) Table 1]. It was also not due to temporal changes in sperm use because this does not seem to occur in mature colonies of *A. echinatior* (21) and because the workers and queens were in any case from the same age cohort. There is therefore a significant genetic influence on queen–worker caste determination in *A. echinatior*. Unlike cases where hybridization or parthenogenesis affect queen–worker determination (22, 23), the genetic influence on caste fate in *A. echinatior* represents standard genetic variation and, unlike the only previous example in the honeybee (11–14), one that is expressed under normal conditions. In addition, the variation is expressed while adult workers have the opportunity to influence larval caste fate, unlike the case of stingless bees where some larvae developing in sealed cells selfishly alter their caste destiny (7, 24, 25). Importantly, most of the queen-biased patrilines in *A. echinatior* also produced at least some workers (Fig. 1), so the genetic influence appears to involve royal genotypes being predisposed to become queens, rather than their destiny being fixed.

The caste system of leaf-cutting ants allows an unusually direct insight into the proximate mechanisms responsible for genetic variation for royalty. Queens, LW, and SW represent distinct size categories (Fig. 1), and, as found previously (16, 17), colonies also showed genetic variation between patrilines in their propensity to develop into LW or SW (SI Table 2). A positive, across-patriline relationship between queen–worker skew and LW–SW skew would indicate a caste-biasing mechanism based on an intrinsic ability to develop into a larger adult. A lack of relationship between the two
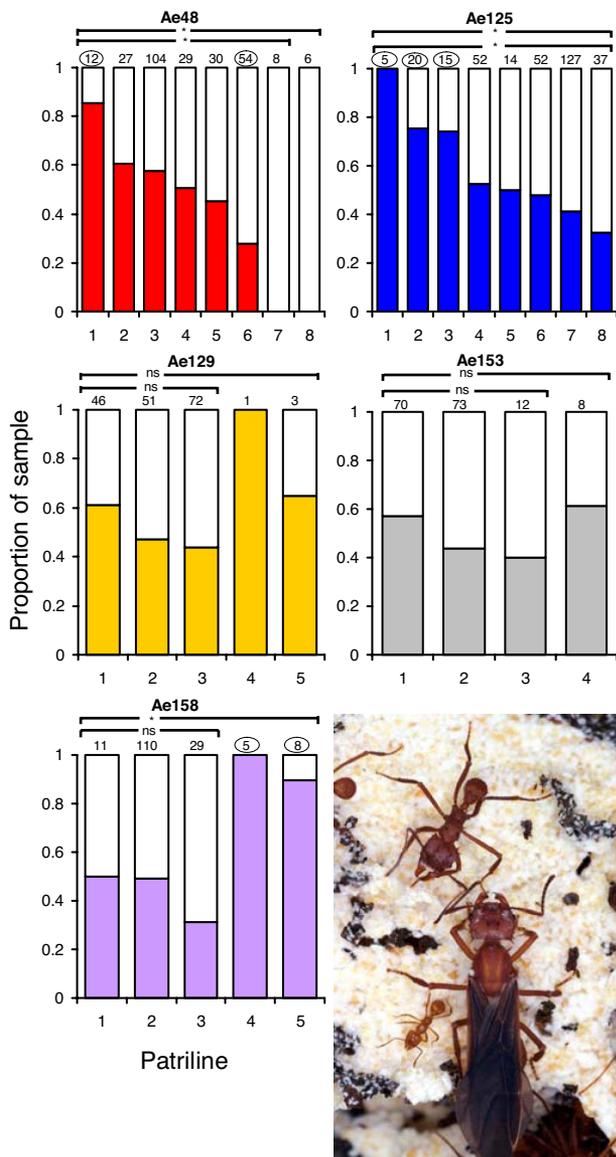
Fig. 1. Genetic influence on queen caste determination in *A. echinatior*. The photo shows the three female castes: small worker, large worker, and queen. Data presented are proportions of individuals sampled per patriline for five colonies that were workers (clear) or queens (colored). The significance of Fisher's (*Top* bars) and *G* tests (*Bottom* bars) are shown testing the null hypothesis that patriline ratios did not vary significantly within a colony (*, $P < 0.05$; ns, $P > 0.05$). Sample sizes are given above the columns and are circled when the individual tests for a patriline showed that its ratio differed significantly from the overall ratio for the colony. In the figure, the worker proportions have been adjusted for the actual numbers of individuals genotyped in each colony such that a proportion of 0.5 is expected for each caste in all colonies.
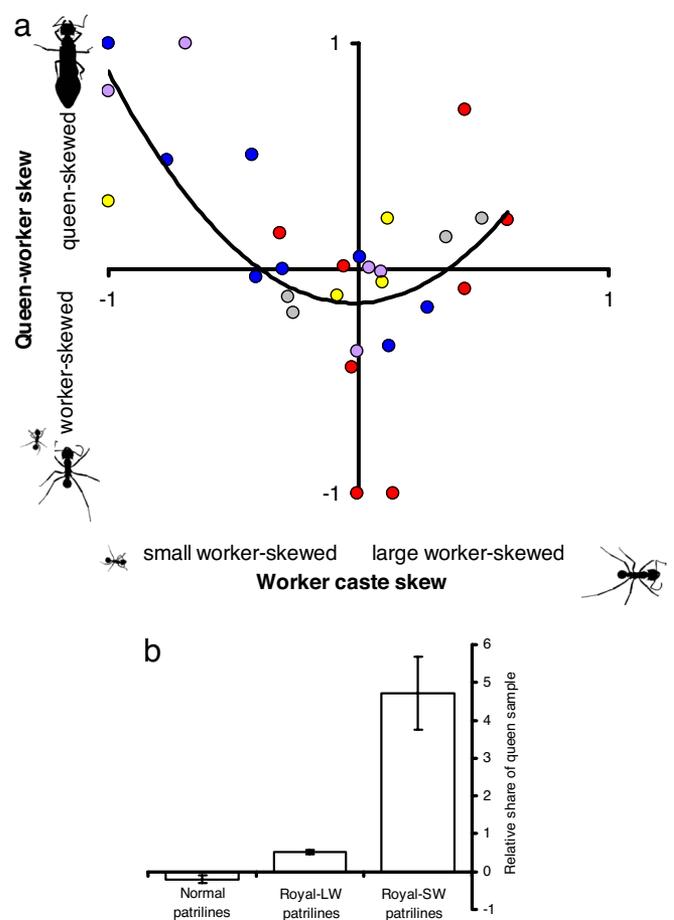

Fig. 2. Relationship between worker caste determination and royalty. (*a*) Each circle represents the skew of a patriline, circle color corresponds to colony colors in Fig. 1, and zero indicates an equal ratio of the two castes. (*b*) Mean ± SEM direct reproductive success of patrilines (share of queen sample relative to share of worker sample, with 0 indicating that the shares were equal) grouped into six royal–small worker (SW) patrilines (above and left of the regression line *x*-intercept in *a*), four royal–large worker (LW) patrilines (above and right of the regression line *x*-intercept in *a*), and the 19 normal unbiased patrilines.

skews would imply that the genetic influence on royalty is decoupled from that on worker caste determination. In fact, the two skews were related but were best explained by a concave ($y = 1.07x^2 + 0.04x - 0.15$, $r^2_{adj} = 0.45$; $F_{2,26} = 10.6$, $P = 0.0004$; Fig. 2) rather than linear function ($F_{1,27} = 2.2$, $P = 0.149$, $r^2_{adj} = 0.04$). Patrilines overrepresented in LW were disproportionately likely to develop into queens, but the same was also true for patrilines that were overrepresented in SW.

The concave relationship between queen–worker skew and LW–SW skew (Fig. 2*a*) indicates that two separate mechanisms are involved in royalty propensity and that both are

linked to worker caste determination. The excess queen production of the patrilines that were also overrepresented in LW rather than SW can be explained relatively easily by a direct genetic influence on adult size. Such an effect would be produced if larvae have a lower response threshold for the environmental cues, such as nutrition, that act at specific thresholds to program the developmental trajectories of larvae (26, 27). Larvae of royal–LW patrilines would then be more likely to develop into a larger adult, i.e., into queens rather than workers and into LW rather than SW (Fig. 3*a*). The relationship between queen-bias and overrepresentation among SW is most probably caused by a similar effect but specifically relating to the queen–worker developmental switch (Fig. 3*a*). Larvae that receive environmental cues appropriate for SW development become SW as normal, but larvae that receive cues appropriate for LW development tend to become queens (Fig. 3*a*). The actual caste proportions observed for the three types of patrilines fit these proposed mechanisms (Fig. 3*b*), the principles of which will also hold for alternative sequences of caste determination to those modeled here (e.g., if queen–worker determination occurs early in development and LW–SW determination occurs later) (27).
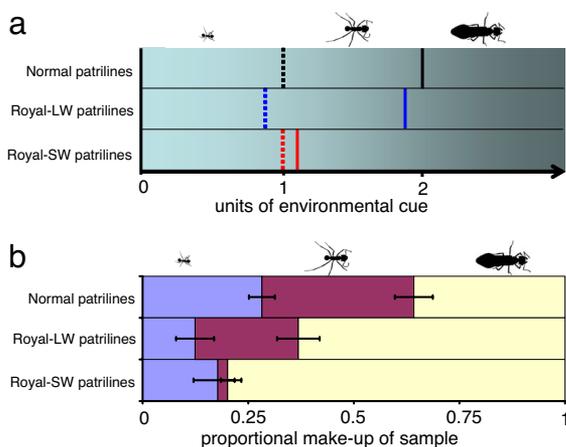
Fig. 3. Mechanisms of royalty biasing. (*a*) Proposed mechanisms of caste determination. Larvae of normal genotypes receiving 1 arbitrary unit of the caste-determining environmental cue (e.g., food) switch from the small worker (SW) to large worker (LW) developmental path (dashed line), whereas larvae receiving 2 units switch from the worker to queen developmental path (solid line). Royal–LW patrilines have lower thresholds for both switches. Royal–SW patrilines have a lower threshold only for the queen–worker switch. (*b*) Mean ± SEM proportion of individuals sampled that were SW, LW, and queens for the four royal–LW patrilines, the six royal-SW patrilines, and the 19 normal unbiased patrilines.

Although both royalty-biasing mechanisms most probably relate to changes in response to caste-determining environmental cues, the contrast between them is important. The mechanism for royal–LW patrilines involves a change in worker caste propensity as well as queen–worker propensity. The trait may therefore plausibly have evolved because of the cooperative benefit of its effect on worker caste determination (16–19), with any selfish benefit from royalty biasing being a pleiotropic side effect or later evolutionary development. However, the change for royal–SW patrilines only appears to affect queen–worker determination and is thus most likely to have arisen primarily for selfish benefits. This distinction is bolstered by the relative direct fitness gained by royal–LW and royal–SW patrilines: the former gain very little direct fitness compared with normal patrilines, whereas the direct fitness of the latter is enhanced by almost 500% (Kruskal–Wallis test: $\chi_2^2 = 18.4$, $P = 0.0001$; Fig. 2*b*). It therefore seems most likely that the genetic disposition shown by royal–SW has evolved for selfish reasons and represents a genuine case of cheating. Further work will be needed to resolve whether genetic variation for cheating traits is discrete or quantitative, as well as whether effects of dominance and epistasis make the degree of expression of cheating genes different depending on the other maternal and paternal genes that are present in a colony.

In addition to predicting that cooperative systems should be vulnerable to cheating, evolutionary theory also predicts that suppression by cooperators will constrain the frequency of royal cheats (2, 3). Our results suggest that this prediction is true for *A. echinatior*. At the population level, significant cheating was limited to 20% of patrilines (6 of 30) in our sample of five colonies. In addition, royal cheats were also rare at the intracolony level (Fig. 4*a*). Most patrilines produced ≈20% of the individuals sampled in their colony, consistent with the average effective mating frequency for *A. echinatior* of 5.3 (15). Royal–LW patrilines had similar frequencies (Fig. 4*a*), but royal–SW patrilines were significantly rarer (Kruskal–Wallis test: $\chi_2^2 = 6.89$, $P = 0.032$; Fig. 4*a*), which suggests that they are constrained by frequency-dependent selection. As in some other systems (4, 6), this selection may result from

group-level efficiency costs or the direct suppression of cheating larvae by cooperative genotypes. Alternatively, the rarity of cheats may be an evolutionary strategy to avoid suppression. Ant larvae signal queen potential at a normally distributed critical size, and adult workers then confirm this potential by feeding the larvae appropriate diet if they assess their signal and size to be congruent with the colony norm (26, 27). Unlike some other social insects (7, 24, 25), workers are therefore potentially able to ignore or eliminate cheating larvae if they detect them, and royal–SW patrilines are especially likely to stand out from the colony norm. Being rare in any particular colony may make them harder to distinguish from the left tail of the size distribution of cooperative queen-potential larvae (Fig. 4*b*). The within-colony rarity of royal–SW patrilines is further confirmation that the trait has evolved primarily for selfish reasons. There is no obvious reason why royal genes that have arisen for cooperative benefits should also be selected to be rare, whereas rarity is exactly what evolutionary theory predicts for royal cheats (2, 3).

Our findings underline that social evolution in very different taxonomic groups can be understood from the same general principles. The nonidentical reproductive interests of group members inevitably result in individual-level selection favoring cheating and the antagonistic coevolution of cheat suppression (1). In systems as diverse as selfish genetic elements (28), worker reproduction in social insect colonies (29) and punishment in human societies (30), suppression by cooperators can prevent cheats being common but cannot eliminate them altogether. Our data confirm this theory for royalty biasing in the leaf-cutting ant *A. echinatior*. As our present results show,
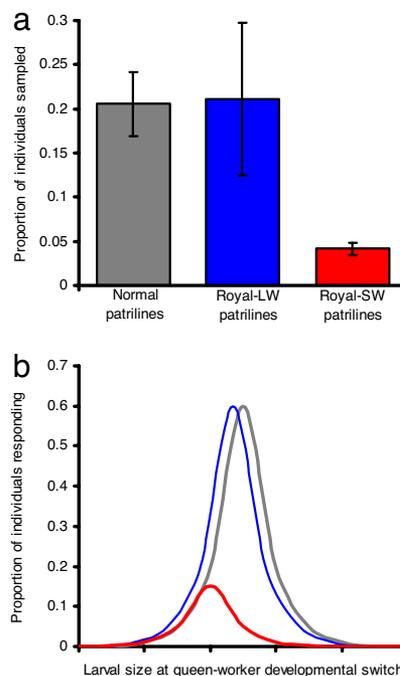

Fig. 4. Intracolonial rarity of royal cheats. (*a*) Mean ± SEM abundance within colonies of the six royal–small worker (SW) patrilines, the four royal–large worker (LW) patrilines, and the 19 normal unbiased patrilines. (*b*) Proposed suppression mechanism that induces rarity. Larvae in normal patrilines (gray) signal queen potential at a normally distributed critical size (arbitrary units) with royal patrilines having slightly (royal–LW, blue) or substantially (royal–SW, red) lower means. Royal–SW patrilines are 75% rarer than other patrilines, which makes their queen-biased larvae harder for adult workers to distinguish from the distribution tail for normal patrilines and allows them to escape suppression.

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cheating at low frequencies can occur in even the most tightly integrated social systems and may thus be more common than presently realized.

Materials and Methods

Workers and virgin queens (gynes sensu stricto) were sampled from five mature colonies of *A. echinatior* that had been collected from Gamboa, Panama. For each colony, workers and queens of similar cuticular coloration were sampled on the same day to minimize age variation. From four of the colonies (Ae125, Ae129, Ae153, and Ae158), 94 queens and 94 workers (split equally between SW and LW) were sampled. From the fifth colony (Ae48), 94 queens have been collected, and a sample of 200 workers from the same collection date has been analyzed previously (16). Queens and workers were genotyped at four polymorphic microsatellite loci: Ech1390, Ech3385, Ech4126, and Ech4225 (31). Allele sizes were scored by comparison with internal size markers. Multilocus offspring genotypes were used to infer the genotypes of colony queens and their multiple mates, and these genotypes were confirmed by using the program MATESOFT, version 1.0 (32). It was then possible to assign the sampled queens and workers to patrilines within their colony with negligible detection errors of patrilines. Individuals whose paternities could not be established because of failed PCR amplification or being heterozygous with the same alleles as a het-erozygous mother queen at one or more diagnostic loci were excluded from the analysis (84 individuals, 7% of total). We determined whether patrilines differed in their ratios of queens to workers by: (*i*) Fisher's exact tests, in which all patrilines were included (33); and (*ii*) *G* tests of heterogeneity, in which the rarest patrilines were excluded such that no more than 20% of cells in the overall analysis for any particular colony had expected frequencies of less than five (34). Both methods examined whether patrilines differed from the total number of queens:workers genotyped for each colony in a uniform direction. Because the analyses involved multiple tests, we controlled for the false-discovery rate by using the program QVALUE (35). Additional worker samples were genotyped for colonies Ae125 and Ae158 to allow the LW–SW skew to be estimated for the two highly queen-biased patrilines.

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