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When can ants discriminate the sex of brood? A new aspect of queen–worker conflict

(*Camponotus floridanus*/brood care/sex recognition/sex ratio/kin selection)

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ABSTRACT The stage in preimaginal ontogeny at which the sexes can first be distinguished has important implications for queen–worker conflict in social insects. If workers are unable to sex larvae at an early instar, their opportunity to control colony reproductive strategies may be limited. In addition, by concealing the sex of her sons for some portion of development, the queen could protect them from the workers' attempts to substitute their own sons or to skew the numerical sex ratio. In a series of choice experiments, workers of the carpenter ant, *Camponotus floridanus*, failed to discriminate the sex of several stages of larvae but did retrieve female pupae significantly more rapidly than male pupae. Our results suggest that in this species, sex may not become detectable until pupation, which is consistent with sexual deception as an aspect of queen control.

The cooperative colonies of the social Hymenoptera are among the principal model systems for the investigation of kin selection and its evolutionary consequences (1). Paradoxically, however, it is the conflicts within these colonies over the source and sex ratio of reproductive offspring that have provided the best tests of the predictions of inclusive fitness theory (2–4). Facultatively or obligately sterile workers obtain indirect reproductive success by rearing offspring for their mother queens, but the optimal goals of queens and workers can differ considerably. Queens can maximize personal reproductive success by monopolizing the production of both sexes, in equal proportions. Workers could enhance their inclusive fitness by biasing investment toward female brood, or if they are able to produce males from unfertilized eggs (5), by replacing the queen's sons with their own.

The ability to differentiate between male and female offspring is an implicit assumption that underlies all models and empirical tests of conflict between queens and workers. Surprisingly in view of its central theoretical importance, brood sex recognition by social insects has been little studied (6). In particular, no experiments have been explicitly designed to determine the stage in preimaginal ontogeny at which the sexes can first be distinguished. The timing of detectable sexual differentiation could have two novel implications for queen–worker conflict. First, uncertainty in identifying young female larvae may constitute an evolutionary constraint, limiting worker options for controlling sex ratio to the skewing of energetic investment late in development. Second, the queen may be able to retain control of male parentage for herself, despite the workers' preference for their own sons, by concealing the sex of her male offspring for some portion of development (7). By effectively “cross-dressing” young males in the pheromones of the opposite sex, the queen could protect them from worker attempts to replace them or to skew the sex ratio. As the males approach

adulthood, the disguise must become untenable, but by then the workers can gain more inclusive fitness by continuing to care for the queen's nearly grown sons than by substituting their own eggs and starting over.

This “sexual deception” hypothesis (7) predicts that, in species whose workers are not obligately sterile yet whose queens dominate male production, young male and female larvae should be virtually indistinguishable to the workers. To test the prediction, we conducted a series of choice experiments to determine whether workers of the carpenter ant, *Camponotus floridanus*, discriminate the sex of brood at different developmental stages. The results suggest that sex becomes detectable only at pupation and are consistent with the concealment of young males as an aspect of queen control.

METHODS

Colonies of *C. floridanus* were reared from founding queens collected in the Florida Keys and were maintained in the laboratory in test-tube nests (8). As is typical of carpenter ant species, colonies contain a single queen whose presence inhibits worker ovarian activity. Approximately 60 days after queen loss or removal, major workers usually begin laying male eggs (ref. 9; N.F.C., unpublished results). Larvae and pupae assumed to be female were obtained from three large (>2000 workers), healthy source colonies with queens. Since the source colonies produced only workers before, during, and after this study, they were unlikely to contain male brood. We obtained male larvae and pupae from three queenless groups, of which two were subcultured from two of the same source colonies and one was derived from a fourth source colony. These groups were initially broodless, and all of the brood that was eventually produced by the disinhibited workers developed into males. Five additional colonies containing 150–250 workers, all unrelated to the male and female brood source colonies, provided test workers for the sex discrimination trials.

In each trial series, a tube from a test colony, containing approximately 20 workers and their queen, was emptied of brood and transferred to a 22 × 30 cm arena. In each trial, a size-matched pair of male and female brood of the same developmental stage was presented 10–15 cm in front of the tube entrance. Four developmental stages were tested: “small” larvae (in the first or second instar), “medium” larvae (roughly half the length of last-instar larvae), “large” larvae (in the final instar), and pupae. (*Camponotus* probably has 5 larval instars; D. Wheeler, personal communication.) Depending on the availability of brood, one or all stages were presented in a given trial series with a single test colony. The average series consisted of 18 trial pairs (range 8–37), but no more than 15 pairs of any one developmental stage were presented in one series. At the end of a series, all retrieved brood were removed from the test tube, which was returned to its colony.

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We recorded the time elapsed from placement of the brood pair until each item was initially picked up (lifted clear of the ground) and until each was retrieved inside the test tube (8). Trials were terminated at 5 min after brood placement; if neither had been picked up, the trial was discarded. If one of the pair was not picked up, its pick-up time was arbitrarily recorded as 5 min, and the trial was not included in the retrieval data. Brood that had been picked up but not yet carried inside the test tube at the end of the trial were scored as retrieved at 5 min. For analysis, the pick-up time for each brood individual was subtracted from its retrieval time to make retrieval preferences independent of pick-up preferences, and all times were logarithmically transformed. We also noted whether brood were dropped during retrieval. A "drop" was scored when a larva or pupa which had been picked up was released and left unattended for at least 30 sec.

Experiments of similar design, in which test workers chose between kin and nonkin worker pupae, have demonstrated that *C. floridanus* preferentially pick up and retrieve related female brood (10). To test the possible involvement of kin recognition in the treatment of male brood, we also presented workers from two source colonies with related and unrelated male pupae, following the protocol described above. These males came from four queenless worker groups, two of which were subcultures of the two source colonies, so that the related males were nephews of the tested workers. Treatment trials used male pupa pairs of which one was related to the test workers and the other was unrelated. As controls to determine any differential attractiveness of the four male kin lines, we presented pairs of male pupae which were both unrelated to the test workers.

RESULTS

Workers exhibited no significant preference for either sex until after the brood had pupated (Fig. 1). There was no indication of discrimination by sex in the pick-up or retrieval times for small, medium, or large larvae, nor for all larval size classes combined, representing a total of 460 choice tests. For no comparison was the significance value less than 0.25. However, while there was no sex bias in the time elapsed to pick-up for pupae, female pupae were retrieved significantly more rapidly than males (matched-pair *t*-test, $t = 3.42$, $df = 235$, $P = 0.001$; this result has a tablewide probability of occurring by chance of $\alpha = 0.008$; ref. 11). Brood were not always retrieved directly into the test tube. In some cases they were picked up, carried away from the tube, and dropped, perhaps indicative of rejection or difficulty of handling. Small larvae were particularly likely to be dropped.

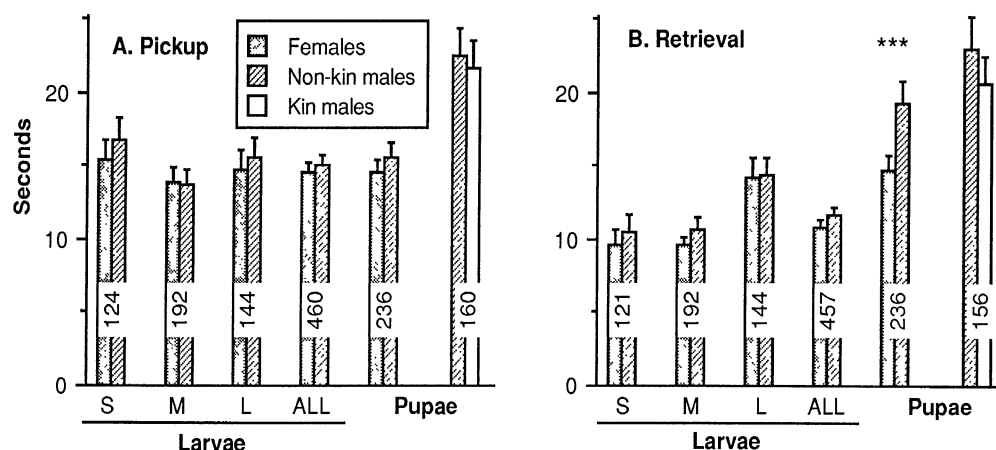


FIG. 1. Average number of sec (+ SE) to pick up larvae or pupae (A) and retrieve them (B). Larvae sizes were small (S), medium (M) or large (L). All female larvae and pupae were unrelated to the adult workers used in the tests. All comparisons were nonsignificant by both matched-pair *t* tests and Wilcoxon signed-rank tests (all $P > 0.25$), except where noted by *** ($P = 0.001$). (Sample sizes for each comparison are displayed at the base of the bars.)

Table 1. Number of trials in which larvae and pupae were dropped after being picked up (n = total number of presented pairs)

	♂	♀	Kin	Nonkin	n	Significance level
Sex discrimination						
Pupae	12	2	—	—	236	$\chi^2 = 5.79$, $P < 0.025$
Large larvae	4	5	—	—	144	NS
Medium larvae	8	3	—	—	192	NS
Small larvae	12	12	—	—	124	NS
Kin discrimination						
Male pupae	—	—	8	12	160	NS

χ^2 tests (with Yates correction) were used for all statistical comparisons. NS, not significant.

The pattern of trials in which brood were dropped was consistent with the retrieval results (Table 1). There were no sex differences in dropping of any larval size class, but male pupae were significantly more likely to be dropped than were females.

When the data for each colony were analyzed separately, there was some indication of idiosyncratic variation in the attractiveness of pupae from different sources. Female pupae from one of the three source colonies were not retrieved significantly earlier than males, and male pupae from one of the three queenless groups were not significantly discriminated against. This variation may suggest that, even at this late stage in development, the recognition of sex is not always easy for the workers. Conversely, the responses to larvae were consistently insignificant, with no idiosyncratic preferences or avoidances for any of the lines of male or female larvae.

No kin recognition was revealed by offering related and unrelated male pupae. The former were neither picked up nor retrieved significantly faster (Fig. 1), and the latter were not significantly more likely to be dropped (Table 1). The control trials again revealed that one of the test colonies significantly discriminated against the males from one unrelated queenless group, irrespective of the source of the other male pupa. However, removal of all trials involving that male source colony from the data set did not significantly alter the overall conclusion. Both pick up and retrieval times were longer when two male pupae were presented than when male and female pupae were paired, though only the former difference was significant ($t = 3.742$, $df = 394$, $P < 0.001$ for pick up, $t = 1.503$, $df = 390$, $P > 0.1$ for retrieval). Possibly the larger range of brood sizes used in the two-male experiments required more assessment and handling time; only small male

pupae were used in the male–female experiments to match the size of the worker pupae.

DISCUSSION

Our results demonstrated significant discrimination of brood sex by *C. floridanus* workers only after the brood had pupated. As always in recognition experiments, an essential caveat must be stated: failure to discriminate does not demonstrate failure to recognize. Workers might be able to identify the sex of very young larvae but, for whatever reason, choose not to treat them differently. However, it is not obvious why workers should behave without bias toward recognizable male larvae and then begin discriminating against them as pupae.

Male pupae were retrieved significantly more slowly than females and were more often dropped and left unattended. It is possible that the workers prefer female pupae because, in a natural *C. floridanus* colony, they would be more closely related to the average female than to the average male (2). However, kin recognition does not seem to be an important aspect of this system, given that workers failed to distinguish between related and unrelated male pupae. Discrimination against males might also result from the workers' perception of them as unfamiliar. The test workers were unlikely to have encountered this sex before, since their colonies were far too small to begin producing males. Thus, the observed bias may occur either because workers recognize male pupae as male and prefer females or because they find female brood more generally similar to their familiar nestmate brood. [This ambiguity is analogous to that arising in some kin recognition experiments, in which mechanisms for rejecting aliens may yield apparent kin preference as a side effect (12, 13).] Since our goal was to establish whether any differences exist between the sexes to which workers might respond, it was not essential (and probably would be difficult to demonstrate) that the workers recognize maleness *per se*.

Assuming that the late development of characteristics identifying sex proves to be widespread in ants and other social Hymenoptera, two novel and not mutually exclusive consequences for queen–worker conflict can be considered. First, worker options in controlling reproductive sex ratios may be more restricted than has been previously appreciated. Empirical evidence, in the form of dry weights of adult alates, generally supports the theoretical contention that ant colonies containing one singly inseminated queen should exhibit a 3:1 female bias in reproductive investment (2, 3). Yet this bias is a characteristic of size-dimorphic sexes; numerically, sex ratios in most species are close to 1:1 (3). Why emphasize the allocation of dry weight rather than the number of dispersing copies of one's genotype produced (2)?

Uncertainty about the sex of brood might make preferential treatment of younger female larvae impossible, so that workers cannot bias the numerical sex ratio. If constrained to rear both sexes in equal numbers, workers can only attempt to readjust investment ratios according to relatedness asymmetries by differential feeding of females late in development. In many ant species, female alates are conspicuously fattened after eclosion, while males may lose weight prior to the mating flight (14, 15). Such size-dimorphic investment biases are much more common in species with one queen per colony, in which queen–worker conflict is expected to be most severe, than in species with multiple queens and weaker queen–worker conflicts (3, 16).

Second, uncertainty about the sex of brood could enhance queen control of male parentage. Though kin selection theory predicts that workers prefer their own sons to those of the queen (17), workers seem rarely to replace queen eggs with their own (5). In genetically heterogeneous colonies, containing multiple or multiply inseminated queens, worker

oviposition may be prevented by mutual “policing,” in which each worker prevents others from reproducing because she shares more genes with the average queen-laid egg than with the average worker-laid egg (7, 18, 19). However, this inclusive fitness argument does not apply to colonies containing one singly inseminated queen, in which any worker's son is more closely related to all workers than any queen-laid male egg. Alternatively, Nonacs (7) proposes that queens can maintain control of male parentage by means of sexual deception.

If a male costs X amount of resources to raise, then from the viewpoint of a nonlaying worker, the values of queen-produced males and worker-produced males are $0.25/X$ and $0.375/X$, respectively. (The values are weighted by the average relatedness to the focal worker.) If, however, an investment of x must go into a queen-laid larva before it is old enough to be recognized as a male, then the worker faces a choice: finish raising that male (whose remaining cost is $X - x$) or start over with a worker-laid egg (which will still cost X). The two types of male will have the same inclusive fitness values for the worker when $x = X/3$ —e.g., if sex cannot be detected until one-third of development is complete. This point shifts to earlier in development if, in attempting to replace males, workers sometimes mistakenly destroy female larvae (7).

By the pupal stage, a *C. floridanus* male has probably received more than one-third of the total energy to be invested in him. Thus, for this species, the apparent absence of both discrimination of larval sex and of worker laying in the queen's presence is consistent with the sexual deception hypothesis. Had we found discrimination by sex from the first instar, the hypothesis would have been falsified, though of course failure to contradict sexual deception does not constitute proof of its existence. Stronger evidence would be provided by comparisons across a range of species, correlating the time at which brood sex becomes detectable with the occurrence of worker reproduction. Unfortunately, available information on this question in other social Hymenoptera is limited.

Concealing males would be of little value when workers police one another or are obligately sterile. In the former case, queens and workers have a common interest in the early detectability of males. In the latter, there is no conflict over male parentage, and the queen could not use sexual deception to win the conflict over investment ratios (because once older males become distinguishable, the workers can still skew the distribution of resources against them). In two such species, brood sex seems to be apparent early in development. Workers of the ant *Monomorium pharaonis*, which lack ovaries, can reportedly identify the sex of eggs (20). The ability of honey bees (*Apis mellifera*) to discriminate the sex of larvae, even when placed in cells of identical size (21), is consistent with evidence of mutual worker policing (22). On the other hand, the distinctiveness of young males (as eggs or larvae) in three other species—the stingless bee *Trigona postica* (23), the bumblebee *Bombus terrestris* (24), and the ant *Myrmica rubra* (25)—is consistent with frequent oviposition by workers in the presence of robust queens. Our findings in *C. floridanus* provide evidence that indistinctiveness of young males is conversely associated with inhibition of worker oviposition in the queen's presence. This correlation, if confirmed by examination of additional species, would support the hypothesis that delaying detectability of brood sex helps queens to control worker reproduction.

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