

Research article

Are workers of *Atta* leafcutter ants capable of reproduction?

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Summary. Workers of most eusocial Hymenoptera can produce sons after queen loss, which (posthumously) benefits the queen and increases worker inclusive fitness. However, the evolutionary loss of worker ovaries has occurred in several lineages, while workers in other taxa may be infertile despite having ovaries. Workers of *Atta* leafcutter ants only lay trophic eggs in queenright colonies. Although *Atta* colonies are commonly kept at universities, museums, and zoos, no reports of worker sons in orphaned colonies exist, suggesting that *Atta* workers are infertile. To explicitly test this, we created eleven orphaned laboratory nests of *Atta cephalotes*, *A. sexdens*, and *A. colombica*, and maintained them for 3–6 months after queen loss. Eight colonies did not produce any brood, but three nests produced 1–4 worker-derived male larvae and pupae. Microsatellite genotyping indicated that these were worker sons. However, all males were tiny (3.5–9 mm long) compared to normal queen sons (16 mm long), and would almost certainly be unable to mate. We also found reproductive eggs, but most of these had no yolk and were thus inviable. We conclude that *Atta* workers are not completely infertile, but that worker fertility is low compared to the sister genus *Acromyrmex*, where workers routinely produce normally-size males after queen loss in the laboratory. We hypothesize that worker reproduction in orphaned *Atta* field colonies is almost never successful because the last workers die before their sons can be raised to adulthood, but that the importance of worker-laid trophic eggs for queen feeding has precluded the evolutionary loss of worker ovaries.

Keywords: Dwarf males, worker sons, orphaned colonies, worker sterility, worker infertility.

Introduction

In the majority of eusocial Hymenoptera with physical female castes, workers are unable to mate because they lack genitalia and a functional spermatheca, but typically possess ovaries and can lay unfertilized eggs that develop into males (Choe, 1988; Bourke, 1988). The contribution of the workers to the parentage of the males under queenright conditions (i.e., in the presence of a healthy mother queen) may range from zero to the majority of the males, depending on taxonomic group and species (Hammond and Keller, 2003; Wenseleers and Ratnieks, submitted). In species where worker sons are rarely produced in queenright colonies, a major proximate cause of the absence of worker sons is typically the low proportion of reproductive workers (Dijkstra et al., 2005; Wenseleers et al., 2004). The tendency of workers not to form reproductive eggs in the presence of the queen is most frequently explained as an adaptive response to an evolutionary history of efficient queen- or worker policing (Ratnieks, 1988; Wenseleers et al., 2004a, 2004b).

Worker reproduction is always detrimental to the fitness of the queen as long as she is alive, because the raising of worker sons reduces the amount of colony resources available to her own sexual offspring. However, the ability of workers to reproduce after the queen's death is expected to benefit the deceased queen as well as the workers (Ratnieks, 1988; Franks et al., 1990), and to carry no real costs as the colony is normally doomed. However, obligate worker sterility (i.e., the complete absence of ovaries in workers) is reported to occur in at least one genus of stingless bees (*Friesomelitta*: Boleli et al., 1999) and nine genera of ants (*Anochetus*, *Eciton*, *Hypoponera*, *Leptogenys*, *Monomorium*, *Pheidole*, *Pheidologeton*, *Solenopsis*, *Tetramorium*: Wheeler, 1910; Oster and Wilson, 1978; Hölldobler and Wilson, 1990; Villet et al., 1991; but see Whelden, 1963 for *Eciton*), and it is likely that more examples will be discovered in the future.

Natural selection will only favor obligate worker sterility when four conditions are simultaneously met: 1. The ontogeny and/or maintenance of ovaries is costly and reduces the ability of workers to work for the colony; 2. Workers do not use trophic eggs to feed the larvae or the queen; 3. Attempted worker reproduction is never successful in the presence of the queen; 4. Workers do not benefit from laying reproductive eggs after queen loss, either because the development time of the males is long compared to the life-span of the workers (so that the last workers in the colony are likely to die before any workers sons can be raised to adulthood), or because gynes are only present in the population during a short mating season (so that worker sons from orphaned colonies are unlikely to find a mate). Factors that are expected to further facilitate the evolution of obligate worker sterility are polygyny, a long life-span of queens compared to workers, and the presence of a mechanism for the superseding of senescent queens and/or “emergency replacement” of dead queens (cf. Hatch et al., 1999; Faustino et al., 2002), because these reduce the probability that a worker will become orphaned during her life-time. A high degree of queen-worker dimorphism is expected to be another facilitating factor, because this would tend to constrain worker fecundity and thus reduce the pay-off of for worker reproduction.

The occurrence of obligate worker sterility in a given species does not reveal much about the evolution of this trait, as in such a species it is impossible to test how unsuccessful worker reproduction would be after queen loss. A better understanding of the evolution of obligate worker sterility may be gained from species in which workers have retained their ovaries, but never succeed in reproducing after queen loss (hereafter: “infertile workers”). An example of infertile workers is found in the Argentine ant *Linepithema humile*, where workers can lay trophic eggs but apparently never reproduce in orphaned colonies (Aron et al., 2001; S. Aron, pers. comm.).

Another candidate taxon for infertile workers are *Atta* leafcutter ants, which are characterized by a high degree of queen-worker dimorphism and long-lived queens (up to 20 years: Weber, 1972; Hölldobler and Wilson, 1990). While most *Atta* workers never develop their ovaries in queenright colonies, a minority of the workers have very specialized ovaries which they use to produce trophic eggs to feed to the queen (Dijkstra et al., 2005). Although *Atta* display colonies are commonly kept at universities, museums, and zoos, to our knowledge only a single dubious report (Tanner, 1892, cited in Wheeler, 1903; see Discussion) of worker sons from orphaned *Atta* colonies exists. However, this lack of evidence is inconclusive, as queenright *Atta* colonies likewise reproduce only very rarely in the laboratory.

The aim of this study was to determine if *Atta* workers are indeed infertile, as anecdotal evidence suggested (Bazire-Bénazet, 1957; Hernandez, 1998; Dijkstra et al., 2005). We compare our results with those obtained from its sister genus *Acromyrmex* (Wetterer et al., 1998), where worker sons are routinely produced in orphaned colonies in the laboratory. Identifying the constraints on worker reproduction in *Atta* may help understand the evolution of obligate worker sterility in other taxa.

Materials and methods

Studying worker reproduction in leafcutter ants

Leafcutter ants are obligately dependent on cultivating a clonally propagated fungus inside their nest as food. Larval and adult ants eat fungal mycelium, as well as clusters (“staphylae”) of modified hyphal tips (“gongyliidia”) (Hölldobler and Wilson, 1990). A problem with studying worker reproduction in *Atta* is the enormous size (millions of workers and hundreds of fungus gardens) of reproducing colonies in the field. Because our laboratory *Atta* colonies were far smaller than mature field colonies, our criterion for successful worker reproduction was the presence of worker-derived eggs and first or second instar larvae (hereafter: “young larvae”), rather than final instar larvae, pupae, or adults.

In *Acromyrmex* leafcutter ants (which have much smaller colonies than *Atta*), queenright laboratory colonies only produce sexuals when the total volume of the fungus garden(s) is above a certain threshold (Bekkevold and Boomsma, 2000), but orphaned laboratory colonies can contain worker-derived male eggs and young larvae at any fungus volume (Dijkstra, 2005). Orphaned *Acromyrmex* workers typically raise a large proportion of the worker-derived brood into adult males with normal size and morphology between 14–40 weeks after queen loss, irrespective of time of year, even if the fungus garden volume is well below the threshold for reproduction by queenright colonies (Dijkstra, 2005). However, orphaned laboratory colonies of *Acromyrmex* leafcutter ants may also fail to produce any adult worker sons if the fungus garden is extremely small, resulting in the starvation of the larvae in the final instar. Starving *Acromyrmex* larvae are not cannibalized by the workers, but dumped intact on the rubbish heap outside the main nest, where they can be easily observed and counted.

Maintenance of orphaned nests

We divided a total of 10 queenright laboratory colonies of *Atta sexdens* ($n = 2$), *A. cephalotes* ($n = 3$), and *A. colombica* ($n = 6$) into orphaned and queenright nests, by moving 1–2 fungus gardens with a median total volume of 1000 ml (range 800–1900 ml) into separate nest boxes. Before division, the queenright colonies consisted of 1–4 fungus gardens with a median total volume of 2900 ml (range 1750–4000 ml). All colonies had been collected in Panama, except for two *A. cephalotes* colonies (CE-T1 and CE-T2) from Trinidad. We maintained the orphaned and queenright nests as separate colonies in the dark, at 25 °C and 70% RH. Further details of the colony set-up and feeding regime are given in Dijkstra et al. (2005). We regularly inspected the surface of the fungus garden and the rubbish heap for adult sexuals and discarded final instar larvae, respectively. Six months after colony division, we killed the orphaned nests by freezing the fungus at –20 °C. An exception was the orphaned nest of CE-T2, which was killed three months after colony division. We also froze an additional *A. cephalotes* nest from Panama (CE-P3), in which the queen had suddenly died four months earlier. We completely dissolved the fungus in 70% ethanol by gentle stirring. Workers, fungus, and fungus substrate tended to remain suspended, while brood tended to settle on the bottom. We took ca. 200 ml aliquots of the fungus suspension, and carefully searched the bottom layer for any eggs, larvae, or pupae under 6.4x magnification against a dark background. We repeated this procedure until the entire fungus suspension had been searched, which typically took 1–1.5 h per nest. The sex and caste of *Atta* brood can be easily distinguished from the final larval instar onwards, and their pupae are not enclosed in cocoons.

Microsatellite genotyping

We found brood in two orphaned *A. colombica* nests (see Results). For these two nests, we genotyped all larvae and pupae ($n = 6$) with two microsatellite loci (*Atta5-6TF* and *Atta7-8TF*: Fjerdingstad et al., 1998), and reconstructed the genotype of the queen from 30 workers (15 small

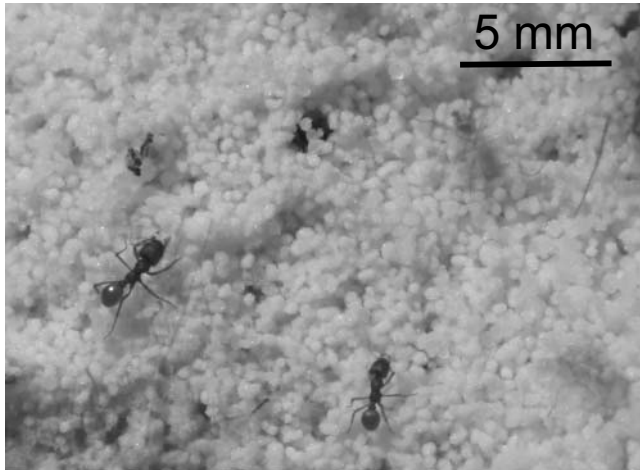


Figure 1. The fungus garden of an orphaned nest of *Atta sexdens*, with two small workers on top of an unusually dense layer of “staphylae” (clusters of modified hyphal tips that function as ant food).

workers and 15 medium workers) per nest. We calculated the effective mating frequency of the queen (m_e ; corrected for binomial sampling error of workers) following formula (2) in Pamilo (1993). We calculated the probability of detecting a worker son (P_d) with formula (2) in Foster et al. (2001). Details about DNA extraction, PCR protocols, and allele scoring are given elsewhere (Fjerdingstad et al., 1998; Hughes et al., 2003).

Results

The last new workers (derived from queen-laid eggs) pupated in the orphaned nests between 5–8 weeks after colony division, which is 4–16 weeks earlier than in *Acromyrmex* (Dijkstra, 2005). The primordium of a fungus fruiting body appeared at the top of the fungus garden in one nest (CE-P3) two weeks after the estimated time of death of the queen, but disappeared one month later without having developed further. In six out of ten orphaned nests (at least one per species), rotting fungus started to accumulate at the bottom of

the fungus gardens within the first two months after colony division. We successfully contained these infections by immediately removing the affected parts of the fungus. In each orphaned nest of *A. sexdens* and *A. cephalotes*, an unusually dense layer of staphylae covered most of the exterior surface of the fungus gardens between 4–6 months after colony division (Fig. 1), but the density of the staphylae did not differ visibly between orphaned and queenright nests of *A. colombica*. Six months after colony division, the median volume of the orphaned fungus gardens was 600 ml (range 25–1000 ml; or 3–100% of the original volume), and about 10% of the workers (median estimate; range <1–25%) were still alive.

One orphaned *A. colombica* nest (CO-3) contained 30 unembryonated eggs, no larvae, two male pupae that were on the verge of eclosing, but no female (worker or gyne) pupae. These males appeared to have a normal morphology (including normal-looking genitalia, ocelli, and wings), apart from their small body size (6.5–9 mm long; see Fig. 2a), which was much less than that of males from queenright field colonies (≥ 16 mm long; see Fig. 1c). Most eggs (80%) in CO-3 were completely transparent due to a lack of yolk and were presumably inviable. Another orphaned *A. colombica* nest (CO-4) contained 44 normal-looking, unembryonated eggs of varying size and shape, two light yellow (ca. 10 d before eclosion) male pupae, one male pre-pupa, one small second instar larva, but no female pupae. The male pupae and pre-pupa were minute (3.5 mm long; Fig. 2b), and had only rudimentary wings, but normal-looking genitalia and ocelli. One orphaned *A. cephalotes* nest (CE-T1) contained a single small second instar larva, but no eggs or pupae of any caste. No eggs, larvae, or pupae of any caste were present in the remaining eight orphaned nests. We never observed discarded larvae or pupae on the rubbish heaps of any of the 11 orphaned nests.

Genotyping revealed that the queens of CO-3 and CO-4 were both highly multiply mated ($m_e = 5.51$ and $m_e = 2.54$, respectively), as is typical for leafcutter ants (Villesen et al., 2003), but with slightly higher paternities than reported earlier for Panamanian *A. colombica* (Fjerdingstad et al., 1998; Murakami et al., 2000). None of the six *A. colombica* (pre)pupae and larvae were heterozygous for either microsatellite locus,

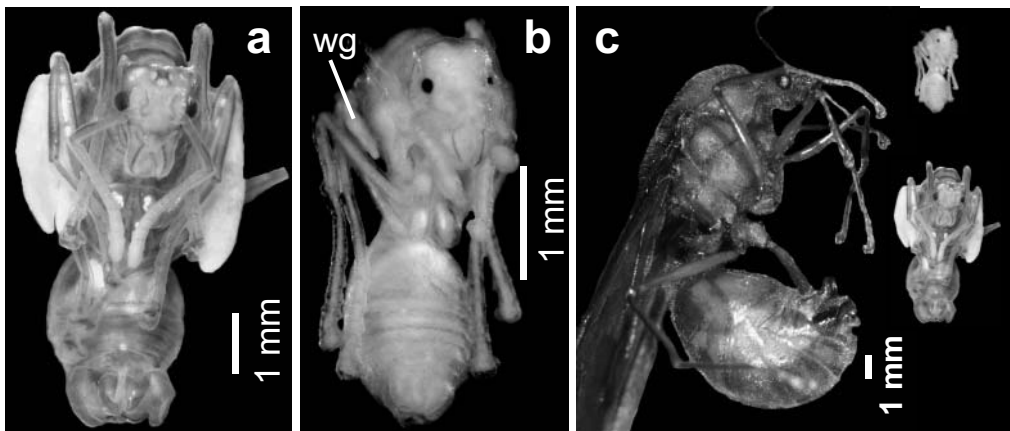


Figure 2. (a) Pupa of a dwarf (6.5 mm) *A. colombica* worker son from orphaned nest CO-3. (b) Pupa of a dwarf (3.5 mm) *A. colombica* worker son with reduced wings (marked “wg”) from orphaned nest CO-4. (c) Normal adult male (16 mm) from a mature colony of *A. colombica* in Panama, with the dwarf males from CO-3 and CO-4 shown at the same scale.

which is consistent with all being haploid males. Three male pupae (50%; one and two in CO-3 and CO-4, respectively) were confirmed worker sons (i.e., were hemizygous for an allele not carried by the queen). Corrected for the probability of detecting worker sons (average $P_d = 0.57$), this is consistent with all six *A. colombica* males being worker sons. The sex and maternity of the single larva in CE-T1 could not be determined due to a lack of microsatellite loci for *A. cephalotes*, but its occurrence six months after queen loss strongly suggests that it was likewise a worker-derived male.

Discussion

Production of dwarf worker sons

The production of dwarf males in two orphaned nests of *A. colombica* was surprising. It is highly unlikely that these males would have any mating success because of the small size of their genitalia, which would not match those of gynes, and because of the incomplete wing development in the smallest male pupae. The small size of these worker sons could be due to maternal effects, for example a lower amount of yolk in worker-laid eggs than in queen-laid eggs, or a reduced attractiveness of worker-derived larvae to nursing workers. Alternatively, their size may have been an artifact of the small size of our laboratory colonies. The latter explanation is consistent with the recent finding of similar dwarf males (presumably queen sons) in a queenright colony of *A. texana* in Dallas Zoo (T.R. Schultz, pers. comm.), as well as a report of “microgynes” produced by queenright laboratory colonies of *A. cephalotes* (Jutsum and Cherrett, 1978). The production of dwarf sexuals by laboratory colonies seems to be an idiosyncrasy of *Atta*, as *Acromyrmex* colonies produce normally-sized sexuals in the laboratory (Dijkstra, 2005).

Mating opportunities outside the mating season

Orphaned workers will only benefit from producing sons when gynes are present in the population by the time that their sons mature. In the field, queenright *Atta* and *Acromyrmex* colonies typically produce sexuals at the beginning of the rainy season (Weber, 1972). However, during the first 2–25 weeks after queen loss, irrespective of time of year, orphaned laboratory colonies of *Acromyrmex* normally produce “posthumous queen offspring”, i.e. gynes and queen sons that are raised from the remaining queen-derived larvae (Dijkstra, 2005). In the field, these gynes would presumably disperse, and have at least some chance of founding new colonies after mating with males from other orphaned colonies outside the optimal mating season. No “posthumous queen offspring” were produced by the *Atta* colonies in this study, nor by a single *A. laevigata* colony (Hernandez, 1998). However, Wheeler (1903) cited a study by Tanner (1892), who reported the production of gynes and males in an orphaned laboratory colony of *A. cephalotes*. Wheeler concluded from these observations that *Atta* workers can parthenogenetically produce

females, and this claim has occasionally been cited (e.g., Ledoux 1974; Choe, 1988). However, the reported timing of eclosion (12–15 weeks after queen loss) strongly suggests that these gynes and males were posthumous queen offspring, making this conclusion untenable.

Low fertility of Atta workers

The presence of worker-derived brood in three colonies (CO-3, CO-4, CE-T1) demonstrates that *Atta* workers (at least in *A. colombica* and *A. cephalotes*) are not completely infertile. However, the high percentage of orphaned nests (72%) that did not produce any brood, as well as the high proportion of abnormal eggs and the low number of young larvae in the remaining nests, suggest that the fertility of *Atta* workers is low. This low rate of worker reproduction is particularly striking when compared to its sister genus *Acromyrmex*, where a similar sample of orphaned colonies kept under the same laboratory conditions gave at least some normal-sized worker sons in all cases (Dijkstra, 2005). The observed accumulation of staphylae in *A. cephalotes* and *A. sexdens* indicate that the near-absence of brood was not simply due to a lack of larval food. Given the low proportion of workers that remained alive six months after colony division, it is unlikely that more brood would have been produced at a later date. In addition, the observed accumulation of rotting fungus in six nests (55%), and the growth of a fruiting body primordium in one nest, imply that waste disposal and fungus tending are inefficient after queen loss.

Evolution of low worker fertility

As explained in the Introduction, obligate worker sterility has evolved in a number of ant genera. Some of these (*Solenopsis*, *Pheidole*, *Tetramorium*) are very species rich, which suggests that obligate worker sterility has been an ecologically successful innovation. We hypothesize that the shorter life-span of *Atta* workers compared to *Acromyrmex* implies that orphaned *Atta* colonies almost never succeed in raising worker sons to adulthood. This constraint will be especially severe under field conditions, where worker survival is predicted to be lower than in the laboratory due to predation, parasitism, and physical wear and tear caused by long-distance foraging. Except for a rare sub-caste of queen nurses with one large “trophic” ovary, queenright *Atta* workers have embryonic ovaries (Dijkstra et al., 2005), which suggests that the ontogeny of ovaries is costly. We speculate that the importance of worker-laid trophic eggs in the diet of the queen (Bazire-Bénazet, 1970; Dijkstra et al., 2005) has been the only factor that has prevented the evolution of obligate worker sterility in *Atta*, as the high egg-laying rate of their giant queens may critically depend on a continuous supply of trophic eggs. The observed pattern of occasional production of worker sons may therefore represent a non-adaptive byproduct of the selection for the maintenance of “trophic” worker ovaries.

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