

Research article

Self-restraint and sterility in workers of *Acromyrmex* and *Atta* leafcutter ants

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Summary. Queens of leafcutter ants (*Acromyrmex* and *Atta*) are highly multiply mated, resulting in a potential queen-worker and worker-worker conflict over who should produce the males in the colony. We studied whether this conflict is expressed, by determining the amount of reproductive egg-laying by workers in queenright colonies of *Acromyrmex echinator*, *Acromyrmex octospinosus*, *Atta cephalotes*, and *Atta sexdens* through ovary dissections. Worker sons are absent or rare in queenright *Acromyrmex* colonies, but can be produced in orphaned colonies. In *Atta*, most workers have rudimentary ovaries that never produce eggs, but a few (mostly small and medium workers that form a retinue around the queen) lay many trophic eggs for consumption by the queen. These eggs are large, flaccid, and lacking in yolk compared to queen-laid eggs, and appear to be always inviable. In *Acromyrmex*, many workers (especially young large workers) lay eggs that are similar in size to queen-laid eggs, but mostly with a reduced amount of yolk. Trophic eggs appear to be an important source of food for larvae in *Acromyrmex* (especially in *Ac. echinator*), but not in *Atta*. Five (0.8%) of 616 dissected *Ac. echinator* workers but no *Ac. octospinosus* workers (n = 552), had ready-to-lay reproductive eggs. Old workers in all four species are incapable of laying eggs due to ovary resorption. We conclude that *Atta* workers are sterile, while *Acromyrmex* workers display reproductive self-restraint, possibly representing an earlier stage in the evolution towards worker sterility. Worker self-restraint in *Acromyrmex* may be maintained by a queen or worker policing mechanism, but individual cost-benefit explanations may also apply.

Key words: Ovarian dissections, trophic eggs, ovary resorption, worker egg-laying, worker reproduction.

Introduction

In most eusocial Hymenoptera, workers can lay eggs but cannot mate. Because of the Hymenopteran haplodiploid sex determination system, worker-laid eggs will develop into males. Reproductive egg-laying by workers results in a potential conflict between workers and the queen over the parentage of the males. Worker egg-laying and worker-derived males are common in orphaned colonies, but in queenright colonies the contribution of workers to the production of males varies greatly between species (Choe, 1988; Bourke, 1988). Of special interest is the parentage of males in species with highly multiply mated queens, e.g. honeybees, some vespine wasps (Foster and Ratnieks, 2000, 2001), leafcutter ants (Boomsma et al., 1999; Fjerdingstad et al., 1998; Fjerdingstad and Boomsma, 2000; Ortius-Lechner et al., 2003; Sumner et al., 2004), *Pogonomyrmex* harvester ants (Cole and Wiernasz, 1999), and army ants (Kronauer et al., in press). A high effective queen mating frequency is a sufficient condition for natural selection to favor worker policing (Ratnieks, 1988), without the need for colony-level productivity costs of worker reproduction (Ratnieks, 1988) or workers preferring a more female-biased colony investment sex-ratio (Foster and Ratnieks, 2001a). In species with worker policing, adult males are predicted to be mainly queen-derived, although workers may still attempt to lay eggs. Worker policing through aggression towards reproductive workers or discrimination against worker-laid eggs has been demonstrated in several singly- and multiply-mated species, e.g. honeybees (Visscher and Ratnieks, 1990), *Vespa* wasps (Foster and Ratnieks, 2001), *Dolichovespula* wasps (Foster and Ratnieks, 2000); *Vespa* hornets (Foster and Ratnieks, 2002); and *Aphaenogaster* ants (Iwanishi et al., 2003).

The total fitness of a worker is the sum of her net contribution to the colony's production of virgin queens, queen sons, and sons of other workers (indirect fitness), and the number of her own sons that reach maturity and are capable of mating (direct fitness), each weighted by the relevant

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relatedness coefficient. Due to various constraints (Cole 1986), reproductive workers are predicted to do less work than non-reproductive workers, resulting in a negative correlation between direct and indirect fitness. Workers are under selection to maximize their total fitness by adjusting their investment in working and egg-laying. Under effective policing, the total fitness may be higher in non-reproductive workers than in reproductive workers because worker-laid eggs are invariably removed, while reproductive workers continue to pay the inclusive fitness price of a lower working ability. Self-restraint may thus be selectively favored as an evolutionary consequence of effective policing, and may explain the near-absence of reproductive workers in queenright honeybee colonies (Ratnieks, 1988). However, self-restraint may also be selectively favored in the absence of policing, e.g. when worker sons mature later than queen sons and therefore have reduced mating success (Duchateau et al., 2004). Possibly as a result of self-restraint, worker egg-laying has secondarily gained a nutritive function in many ants and stingless bees. In these species, workers lay specialized trophic eggs of reduced viability that have a distinct morphology and function as food for the queen or larvae (Hölldobler and Wilson, 1990; Crespi, 1992). In many species, workers can switch from laying trophic eggs to reproductive eggs after queen loss (Crespi, 1992).

Given the ecological importance of the leafcutter ants and the large number of evolutionary studies on this group during the last decade (for a review see e.g. Price et al., 2003), worker egg-laying in this tribe has remained understudied. The only studies available so far are by Murakami et al. (2000), who dissected small numbers of *Acromyrmex octospinosus* workers and reported no fully developed eggs, without giving

further details, and by Bazire-Benazet (1970), who showed that some workers of *Atta laevigata* lay trophic eggs that they feed to the queen. In addition, genetic marker studies have so far failed to find evidence of worker reproduction in queenright colonies of the leafcutter ant *Ac. octospinosus* (Boomsma et al., 1999) and a number of closely related fungus-growing ant genera (Villesen and Boomsma, 2003). The aim of the present study is to determine the frequency and within-nest distribution of trophic and reproductive egg-laying workers in queenright leafcutter ant colonies, and to unravel some of the ultimate and proximate causes of the observed patterns of self-restraint and worker sterility.

Materials and methods

We sampled workers from eight leafcutter ant colonies maintained in the laboratory, two colonies each of *Acromyrmex echinator*, *Acromyrmex octospinosus*, *Atta sexdens*, and *Atta cephalotes*. The colonies were kept in darkness at constant 70% RH and 25 °C. Fungus gardens were kept under inverted plastic beakers inside plastic boxes coated with Fluon™ (ICI Chemicals Ltd.). The colonies received fresh bramble leaves and dry rice twice weekly inside a plastic container, and fresh fruit every two weeks. All colonies had a single functional queen and had been stable in the laboratory for at least six months prior to sampling. Sexuals were produced by the two *Ac. echinator* colonies only. Details of the colonies are given in Table 1.

Leafcutter ants show conspicuous worker size polymorphism, but sub-castes generally do not have discrete size ranges due to the occasional occurrence of intermediate individuals (Wetterer, 1999). In *Atta*, we distinguished large workers (LW, head width >2.70 mm), media workers (MW, head width 1.35–2.69 mm), and small workers (SW, head width <1.35 mm). In *Acromyrmex*, we distinguished only large workers (LW, head width >1.50 mm) and small workers (SW, head width <1.50 mm). Head width was defined as the widest head width in

Table 1. Colonies used in the study. Fungus volume represents the number and size of upturned beakers containing the fungus garden. LW = Large workers, MW = media workers, SW = small workers.

Colony	Species	Origin	Fungus garden volume	Number of dissected workers
Ae145	<i>Acromyrmex echinator</i>	Panama	1 × 1 L + 1 × 0.4 L	190 LW 115 SW
Ae150	<i>Acromyrmex echinator</i>	Panama	2 × 1 L	174 LW 137 SW
Ao35	<i>Acromyrmex octospinosus</i>	Panama	2 × 1 L	169 LW 146 SW
Ao86	<i>Acromyrmex octospinosus</i>	Panama	2 × 1 L	102 LW 135 SW
AtcT1	<i>Atta cephalotes</i>	Trinidad	4 × 1 L	69 LW 156 MW 176 SW
AtcT2	<i>Atta cephalotes</i>	Trinidad	4 × 1 L	56 LW 124 MW 116 SW
Ats1	<i>Atta sexdens</i>	Panama	2 × 1 L	49 LW 132 MW 175 SW
Ats2	<i>Atta sexdens</i>	Panama	2 × 1 L	51 LW 130 MW 182 SW

Table 2. Worker behavioral categories.

Behavior	Description	Location in nest	<i>Acromyrmex</i>	<i>Atta</i>	Relative worker age
Egg care	Carrying and cleaning eggs	Interior queenright fungus garden	SW	MW, SW	Callow, young
Curled up	Resting curled up in fungus, head inwards, legs and gaster outwards	Queenright/queenless fungus garden surface	LW	LW, MW	Young
Larva/pupa care	Feeding, carrying, and cleaning final instar larvae; carrying and cleaning pupae	Queenright/queenless fungus garden surface	LW, SW	MW, SW	Young
Queen nurse	Feeding and grooming the queen; riding on queen's body	Close to queen (queenright fungus garden only)	None	MW, SW	Young
Walking	Building fungus; processing forage; walking on fungus surface	Queenright/queenless fungus garden surface	LW, SW	LW, MW, SW	Middle-aged
Foraging	Foraging on leaves or fruit, protecting foragers	Outside: food box	LW, SW	LW, MW, SW	Old
Waste disposal	Carrying waste, maintaining waste pile	Outside: rubbish dump	LW, SW	MW, SW	Old

dorsal view, excluding the eyes. When sampling workers, we further distinguished seven categories, based on behavior and location in the nest (Table 2). In both genera, workers display an age-graded polyethism, with the oldest workers working outside the fungus gardens. Within each category, we sampled approximately 15 workers at random for each sub-caste. However, not all combinations of species, sub-castes, and categories occur (Table 2). Sampling was done separately for the fungus garden in which the queen was located (hereafter the queenright fungus garden), and one fungus garden from which the queen was absent (hereafter the queenless fungus garden). Workers were able to move freely between fungus gardens. Two behavioral categories (egg care and queen nursing) only occur in queenright fungus gardens. We collected workers from the fungus gardens with forceps and immobilized them on ice. Workers were killed and dissected immediately, or stored at -20°C for later dissection. Comparisons showed that freezing had no effect on the oocytes except to fix the fluid yolk of medium-sized yolk oocytes in arbitrary shapes. We dissected workers by gently pulling the last gastral sternite loose with Dumont INOX#5 forceps, while holding the worker submerged in insect saline solution (ISS; 7.5 g NaCl, 0.35 g KCl, 0.278 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, in 1 L double-distilled water). The ovaries were mounted on a microscope slide with a droplet of ISS and examined at 40 \times or 57 \times magnification under a stereomicroscope. Selected ovaries were also examined in distilled water at 1000 \times magnification with oil immersion under a dark-field compound microscope. For each ovariole, we noted the number of oocytes containing yolk, the degree of resorption of the internal ovariole structure, and the presence or absence of corpora lutea. For each oocyte containing yolk, we scored the length, maximum width, shape, and the presence or absence of a cluster of functional nurse cells. We also classified the color and degree of translucence of the oocyte: colorless and fully transparent (very young non-yolk oocyte); light yellow translucent (young growing oocyte); white translucent (non-fertile, middle-aged to mature oocyte: see figs. 1d-g); and white opaque (fertile, middle-aged to mature oocyte: see figs. 1c,i). Because yolk is opaque white in color, the degree of translucence gives an estimate of the yolk density inside the oocyte. For comparison with oocytes in workers, we also dissected small numbers of *Acromyrmex* mother queens, gynes, callow workers and orphaned workers (Fig. 1). Finally, we measured reproductive eggs from queenright fungus gardens of all four species.

Statistical analyses were performed using JMP v 5.01 for Macintosh (SAS Institute inc., 2002) with the exception of multinomial general linear models (GLMs) which were performed using GLMStat X 5.7.6 for Macintosh (Beath, 2004).

Results

Worker head widths

In *Acromyrmex*, worker head widths were 2.08 ± 0.17 mm (Mean \pm SD, $n = 364$) in *Ac. echinator* LW, 1.25 ± 0.24 mm ($n = 252$) in *Ac. echinator* SW, 2.33 ± 0.29 mm ($n = 271$) in *Ac. octospinosus* LW, and 1.04 ± 0.24 mm ($n = 281$) in *Ac. octospinosus* SW. In *Atta*, head widths were 4.46 ± 0.74 mm ($n=127$) in *At. cephalotes* LW, 1.75 ± 0.25 mm ($n = 486$) in *At. cephalotes* MW, 0.95 ± 0.22 mm ($n = 332$) in *At. cephalotes* SW, 3.56 ± 0.42 mm ($n = 200$) in *At. sexdens* LW, 1.89 ± 0.33 mm ($n = 415$) in *At. sexdens* MW, and 0.87 ± 0.20 mm ($n = 397$) in *At. sexdens* SW.

General morphology of the leafcutter worker reproductive apparatus

Females of leafcutter ants have a pair of telotrophic ovaries with one to several hundred ovarioles per ovary. In telotrophic ovaries, clusters of nurse cells alternate with developing oocytes, with each oocyte lying proximal to its nurse cell cluster. Typical of Hymenopteran telotrophic ovaries is that each nurse cell cluster lies in its own follicle separate from the follicle around the oocyte. In oocytes that accumulate yolk, a bridge of cytoplasm connects the oocyte with the nurse cells via a gap in the follicles. The nurse cells later eject their cytoplasm into the oocyte and degenerate, leaving an empty follicle distal to the oocyte follicle. When an oocyte is ovulated, the ruptured oocyte follicle (corpus luteum) with the empty nurse cell follicle remains, connected to the next oocyte follicle by a ligament of pre-follicular cells (Fig. 1d; see also Minkenberg and Petit, 1985). The corpus luteum then degenerates, may become dark yellow (Fig. 1i), and may be resorbed or extruded with the next laid egg.

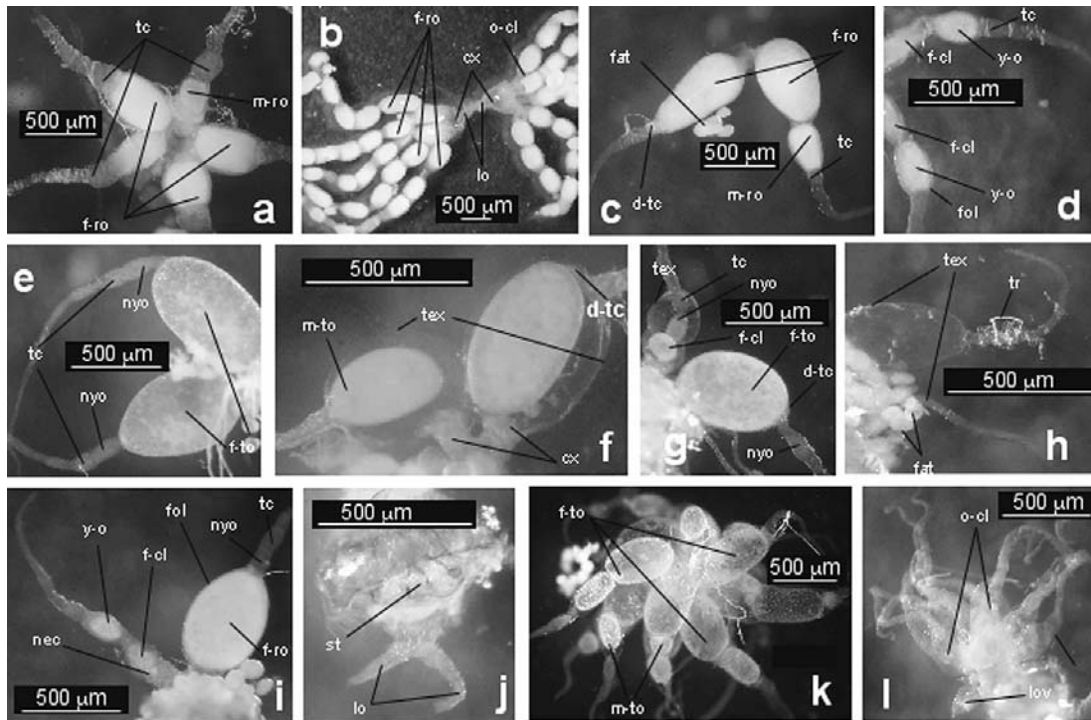


Figure 1. Ovaries of leafcutter ants. a: One ovary of an *Ac. echinator* virgin queen; b: Ovaries of an *Ac. echinator* established mother queen. Note the yellow-coloured debris from breakdown of corpora lutea in the calyces; c: Ovaries of an orphaned *Ac. octospinosus* worker with three reproductive eggs in various stages of development; d: Ovaries of a queenright *Ac. octospinosus* worker that recently laid an egg from each ovariole, as evident from the fresh corpora lutea (ruptured follicles); e: Ovaries of a queenright *Ac. echinator* worker with two ready-to-lay trophic eggs; f: Ovaries of a queenright *Ac. octospinosus* worker with a ready-to-lay trophic egg in the right ovary and a medium-sized trophic egg in the left ovary. Both ovaries are in the first stage of ovary resorption, with the tunica externa separating from the vitellarium and billowing outwards; g: Ovaries of a queenright *Ac. echinator* worker, with a ready-to-lay trophic egg in the right ovary and a fresh corpus luteum proximal to a non-yolky oocyte in the left ovary. The left ovary is in an advanced state of ovary regression; h: Completely resorbed ovaries of a queenright *Ac. echinator* worker; i: Ovaries of a queenright reproductive *Ac. echinator* worker. Note the opaque egg (presumed to be viable) in the right ovary. Part of the corpus luteum in the left ovary is becoming yellow, indicating the onset of corpus luteum breakdown; j: Rudimentary ovaries typical of most queenright *Atta* workers. No ovarioles have yet grown from the lateral oviducts, and the common oviduct and lateral oviducts lack a lumen; k: Trophic ovary of a queenright *At. sexdens* media worker from the queen retinue with 14 ovarioles per ovary. The other ovary is rudimentary and invisible. l: Degenerated left ovary (right ovary in picture) of a queenright *At. cephalotes* worker that formerly laid trophic eggs. The internal ovariole structure has been resorbed, leaving yellow-coloured degraded corpora lutea lying loose inside the ovarioles. The rudimentary right ovary is visible in the lower left corner. cx: calyx; d-tc: degenerated trophocyte cluster; fat: fat cell; f-cl: fresh corpus luteum; fol: follicle; f-ro: full-sized reproductive oocyte; f-to: full-sized trophic oocyte; lo: lateral oviduct; loy: left ovary; m-ro: medium-sized reproductive oocyte; m-to: medium-sized trophic oocyte; nec: necrotic corpus luteum cells; nyo: non-yolky oocyte; o-cl: old (degenerated) corpus luteum; st: sting; tc: trophocyte cluster; tex: tunica externa; tr: tracheoles; y-o: young yolky oocyte.

In all these aspects, leafcutter ants are typical of Hymenopteran females.

Acromyrmex and *Atta* worker ovaries differ in several respects. First, *Acromyrmex* workers eclose with fully formed ovarioles and reproductive tracts, although the ovarioles do not yet contain visible oocytes. In contrast, *Atta* workers have only rudimentary ovaries and reproductive tracts at eclosion (Fig. 1j). In young *Atta* workers, the lateral oviducts, common oviduct, and uterus lack a lumen, and the ovarioles are absent. In older *Atta* workers, the reproductive tract may develop and ovarioles may grow from the distal end of the lateral oviducts. However, in most *Atta* workers the ovaries and reproductive tract appear to develop only slightly and never become functional. Second, ovary size and the stage of development of the oocytes were similar in the left and right ovary of all dissected *Acromyrmex* workers. In ovary-developed *Atta* workers, either the right or left ovary

was developed and contained oocytes, while the other ovary was completely undeveloped (Figs. 1k, l). Third, laying *Atta* workers are more productive than laying *Acromyrmex* workers. Most *Ac. echinator* and *Ac. octospinosus* workers had only a single ovariole per ovary, irrespective of worker size, although a single *Ac. echinator* LW had two ovarioles in one ovary. In contrast, *At. sexdens* and *At. cephalotes* laying workers had 1-16 and 1-9 functional ovarioles per ovary, respectively. For comparison, queens of *Ac. echinator* and *Ac. octospinosus* have 5-6 ovarioles per ovary (M.B. Dijkstra, personal observation), while *Atta* queens may have over 300 ovarioles per ovary (Tschinkel, 1987).

In laying *Atta* workers, the number of functional ovarioles increased significantly with increasing head width (Fig. 2; double log-transformed linear regression: *At. sexdens*: slope = 1.23, $r^2 = 0.62$, $p < 0.0001$; *At. cephalotes*: slope = 0.98, $r^2 = 0.32$, $p < 0.0001$). This means that large *Atta* workers can

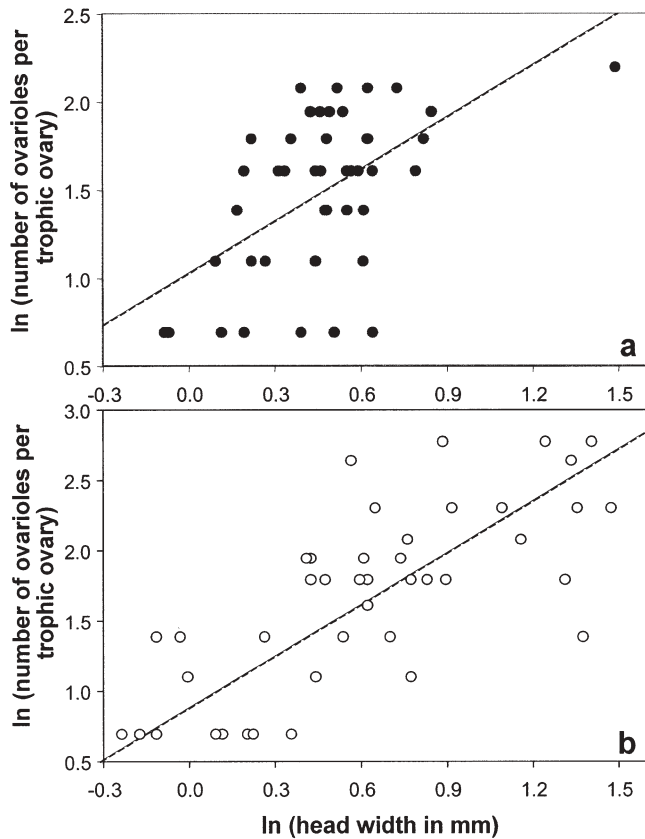


Figure 2. The number of ovarioles per developed ovary increases with head width in (a) *Atta cephalotes* and (b) *Atta sexdens* workers that have a trophic ovary. Both axes have been natural-log transformed.

potentially lay more eggs than small workers. In addition, none of the dissected queenright *Acromyrmex* workers had more than one mature egg per ovariole, while laying *Atta* workers frequently had 2–3 nearly complete oocytes per ovariole. The refractory period between ovulations from the same ovariole is thus likely to be shorter in *Atta* than in *Acromyrmex*. Fourth, the largest *Atta* workers (head width >4 mm) had non-functional spermathecae that are far smaller than those of virgin queens, while smaller *Atta* workers, and all dissected *Acromyrmex* workers, lacked spermathecae entirely.

Comparing worker-produced eggs and eggs from fungus gardens

Once laid, leafcutter ant reproductive eggs are sticky and lie in clumps in the interior of the queenright fungus garden. We assumed that all eggs obtained from the egg piles in the fungus garden were queen-derived, an assumption which has been validated in *Ac. echinator* by genotyping of eggs (M.B. Dijkstra and J.J. Boomsma, unpublished data). In *Acromyrmex*, clumps of 5–40 young eggs are completely surrounded by an envelope of fungal hyphae, although older eggs may be solitary. In *Atta*, egg clumps contained hundreds

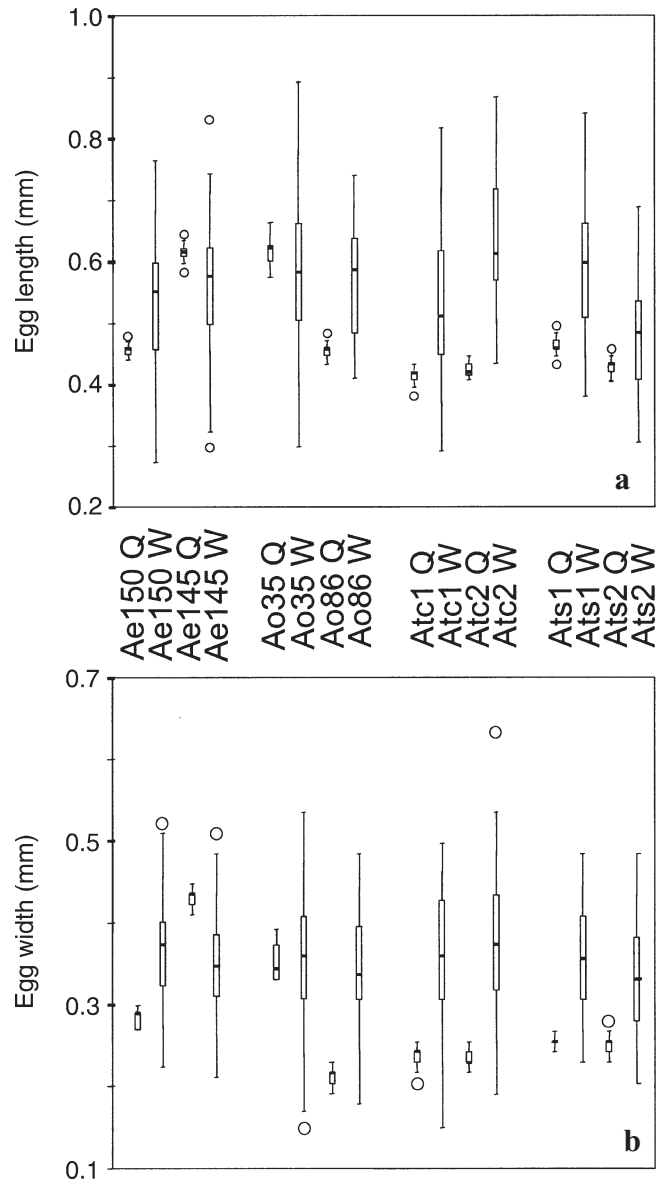


Figure 3. Box plots showing the length (a) and width (b) of eggs from fungus gardens (assumed to be exclusively queen-laid) and of ready-to-lay eggs inside worker ovaries. Bold horizontal bars: median; box contours: 50% quartiles; error bar: 95% confidence intervals; open circles: outliers. Ae: *Acromyrmex echinator*; Ao: *Ac. octospinosus*; Atc: *Atta cephalotes*; Ats: *Atta sexdens*.

of eggs, without a fungus envelope. Eggs from fungus gardens were similar in size within each colony, but highly variable between colonies of the same species (Fig. 3). This indicates that egg dimensions are idiosyncratic and specific for the queens that lay them. In all four species, workers produced ready-to-lay eggs (defined as regularly-shaped terminal oocytes with a fully collapsed nurse cell cluster) of similar size (Fig. 3). To compare the sizes of ready-to-lay worker eggs with queen-laid eggs from fungus gardens, we fitted a General Linear Model (GLM) with egg length as the dependent variable, and species, colony nested within species, and caste of the egg’s mother (worker or queen) as explanatory

variables. Separate analyses were performed for *Atta* and *Acromyrmex*. In *Acromyrmex*, only colony was highly significant ($p < 0.0001$), while all other variables were non-significant (all $p > 0.3$). This means that egg length is highly variable between colonies, but worker- and queen-produced eggs have the same overall length distribution in either *Acromyrmex* species. In *Atta*, both caste and colony were highly significant predictor variables ($p < 0.0001$). This means that *Atta* worker eggs are significantly larger than queen eggs, and that egg length varies significantly between colonies.

Resorption of ovaries

Older workers in all four species tend to resorb the ovariole internal structure, including the oocytes. In the first stage of this process, the ovariole tunica loosens from the vitellarium and billows outward (Fig. 1f). As the elasticity of the tunica is important in ovulation and movement of the follicles down the ovariole, this change implies sterility of the affected ovariole. Subsequently, the developing oocytes and attached corpora lutea are pulled back towards the distal ovariole and shrink (Fig. 1g). In the final stage of this process, only empty tunicae remain (Figs. 1h,l), of which the lumens occasionally contain dark yellow loose-lying corpora lutea. Ovariole resorption appears irreversible and can start irrespective of the developmental stage of the terminal oocytes. Ovariole resorption was not always synchronized between ovaries, or between ovarioles of the same ovary. Ovary resorption appears to be the ultimate fate of all *Acromyrmex* workers. In *Atta* workers, resorption was only observed in developed ovaries. In none of the studied species did we find evidence for resorption of individual oocytes, without parallel resorption of the entire ovariole.

Laying *Atta* workers

Atta workers with a developed ovary produced only large, flaccid eggs that were transparent when at full size due to lack of yolk (Fig. 1k), strongly suggesting that they are trophic and inviable. The eggs were irregularly shaped inside the ovarioles, but assumed a regular spherical shape when dissected out, demonstrating the flexibility of the chorions. The presence of corpora lutea attached proximal to the terminal follicles indicates that the produced eggs are laid. Workers with a trophic ovary were not equally distributed within the colonies (Fig. 4a, b, c). We analyzed their distribution by fitting a full factorial nominal logistic model using the presence or absence of a trophic ovary as the binary response variable, and the factors species, colony nested within species, sub-caste, and behavior as explanatory variables. For greater statistical power and because some behaviors were only carried out by particular sub-castes, we collapsed the behaviors “egg care”, “larval/pupal care”, “walking”, and “curled up” into “other activities”, and collapsed “waste disposal” and “foraging” into “outside behaviors”. The behavior “queen nurse” was retained as a separate category since this is shown only

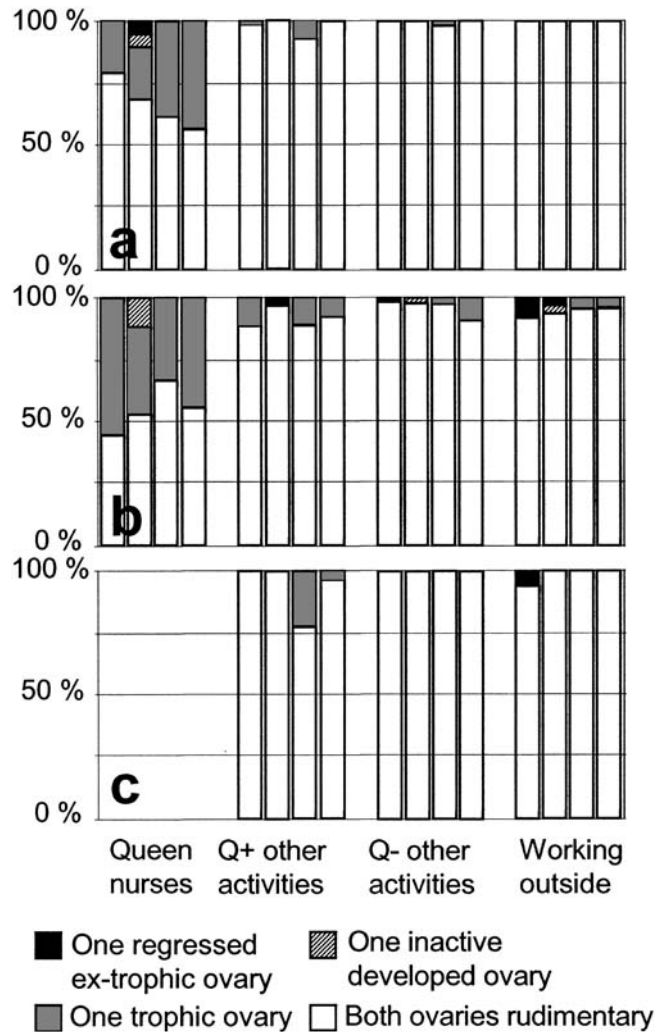


Figure 4. Types of ovaries found among small (a), media (b), and large (c) *Atta* workers performing different tasks. Left to right within each cluster of four bars: colonies AtcT1, AtcT2, Ats1, and Ats2.

in very close proximity to (usually on top of) the queen. However, since this behavior is only shown by SW and MW, we carried out two separate analyses. The first included all sub-castes but excluded the behavior “queen nurse”. This showed a small and insignificant difference between colonies within each species ($p = 0.0922$), with none of the other explanatory variables significant (all $p > 0.9$). This shows that there was no difference between species, colonies, sub-castes, and behaviors (excluding queen nursing) in the probability of a worker having a trophic ovary. The full ANOVA table is given in Table 3a. In the second analysis, we included all behaviors, but excluded LW from the analysis. The full ANOVA table is given in Table 3b. The factor behavior was highly significant, indicating that queen nurses were significantly more likely to have a trophic ovary than workers in all other activities. All other explanatory variables and all interactions were non-significant ($p > 0.1$), indicating that the distribution of trophic egg layers did not differ between *Atta* colonies, nor between SW and MW.

Table 3a. Results of nominal logistic analysis of the probability of *Atta* workers having trophic oocytes present or absent, including all sub-castes but excluding the behavior “queen nurse”.

Source	df	Wald χ^2	P
Species	1	0.0131	0.909
Sub-caste	2	0.00437	0.998
Behavior	1	0.00608	0.938
Sub-caste \times Species	2	0.00178	0.999
Behavior \times Species	1	0.0000734	0.993
Behavior \times Sub-caste	2	0.000961	0.999
Species \times Sub-caste \times Behavior	2	0.00346	0.998
Colony within Species	2	4.77	0.0922

Table 3b. Results of nominal logistic analysis of the probability of *Atta* workers having trophic oocytes present or absent, including all behaviors but excluding the sub-caste “large workers”.

Source	df	Wald χ^2	P
Species	1	0.0168	0.897
Sub-caste	1	0.0175	0.895
Behavior	2	80.6	<0.0001
Sub-caste \times Species	1	0.00524	0.942
Behavior \times Species	2	2.09	0.351
Behavior \times Sub-caste	2	1.73	0.421
Species \times Sub-caste \times Behavior	2	0.291	0.865
Colony within Species	2	3.90	0.142

Laying Acromyrmex workers

Overall, 25% of *Ac. octospinosus* SW, 54% of *Ac. octospinosus* LW, 52% of *Ac. echinator* SW, and 69% of *Ac. echinator* LW produced large, spherical, inflexible eggs with apparently normal chorions. Over 60% of ovarioles with yolky oocytes contained corpora lutea attached proximal to the terminal follicle, showing that *Acromyrmex* workers frequently lay the eggs that they produce. The oocytes in their ovaries varied greatly in opaqueness, apparently due to a continuous variation in yolk density inside the oocytes. A total of five (0.8%) dissected *Ac. echinator* workers, including LW and SW from both *Ac. echinator* colonies, had ready-to-lay eggs that were completely opaque and indistinguishable from those produced by queens and orphaned workers (cf. Fig. 1i). We infer that only these eggs are viable. No completely opaque ready-to-lay eggs were found in *Ac. octospinosus* workers. However, the difference between the two *Acromyrmex* species in the proportions of viable-looking oocytes did not quite reach significance (Fisher exact test, $p = 0.0641$). Most workers in both *Acromyrmex* species produced eggs that were translucent to varying degrees, indicating that they contain less yolk than eggs of gynes, mother queens, and orphaned workers. We assumed all translucent eggs to be inviable or to have reduced viability. We were unable to assign the youngest yolky oocytes to the viable or inviable category.

Workers with yolky oocytes in their ovaries were not equally distributed within the colonies (Fig. 5a,b). We analyzed their distribution by fitting a full factorial nominal

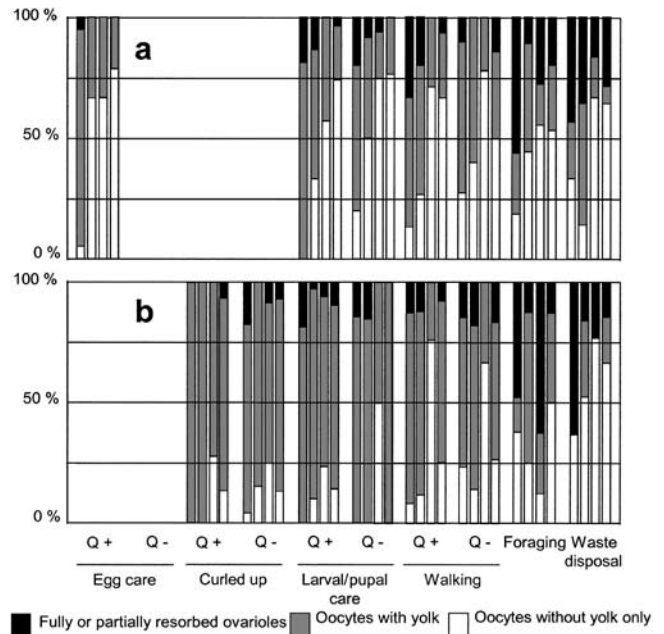


Figure 5. Types of ovaries found among small (a) and large (b) *Acromyrmex* workers performing different tasks. Left to right within each cluster of four bars: colonies Ae150, Ae145, Ao86, and Ao35.

logistic model using the presence or absence of yolky eggs as the binary response variable, and the factors species, colony nested within species, sub-caste, presence or absence of the queen in the same fungus garden as the worker, and worker behavior as explanatory variables. Since some behaviors are sub-caste or location specific (Fig. 5), only ants engaged in activities in or on the fungus garden were included in the analysis, and “curled up” ants were excluded (since this behavior is only shown by LW). This analysis showed a significant difference between species, with *Ac. echinator* being more likely than *Ac. octospinosus* to have yolky eggs present. There were also significant effects of sub-caste, behavior and their interaction, with LW being much more likely to have yolky eggs present than SW, ants caring for eggs or brood being more likely to have yolky eggs than those walking on the fungus garden, and with SW walking on the fungus garden having a further reduced probability of having developed yolky eggs. The full ANOVA table for the analysis is given in Table 4a. Since only LW were found curled up in the fungus garden, the probability of their having developed ovaries was compared with that of the other LW workers confined to the fungus garden (i.e., those tending brood or eggs), also using a full factorial nominal logistic analysis. This showed that there was no significant difference between LW curled up and those tending eggs or brood in their probability of having developed yolky oocytes (Wald $\chi^2 < 0.06$, $p > 0.8$ for all main effects and interactions, Wald $\chi^2 = 1.98$, $p = 0.373$ for colonies nested within species).

Within the fungus garden, most *Acromyrmex* workers have a low probability of showing signs of resorbed ovarioles, while those engaged in foraging or waste disposal outside the fungus garden appear to have a greater probability of

Table 4a. Results of nominal logistic analysis of the probability of *Acromyrmex* workers having yolky oocytes present or absent, excluding the behavior “curled up” (only shown by large workers) and all workers found outside the fungus gardens.

Source	df	Wald χ^2	P
Species	1	22.1	< 0.0001
Queen (presence or absence)	1	0.103	0.748
Sub-caste	1	43.2	< 0.0001
Behavior	1	6.62	0.010
Species \times Queen	1	0.920	0.338
Species \times Sub-caste	1	2.87	0.0900
Queen \times Sub-caste	1	0.124	0.725
Species \times Behavior	1	0.148	0.701
Queen \times Behavior	1	0.0000236	0.996
Sub-caste \times Behavior	1	7.92	0.0049
Species \times Queen \times Sub-caste	1	0.919	0.338
Species \times Queen \times Behavior	1	0.156	0.693
Species \times Sub-caste \times Behavior	1	0.845	0.358
Queen \times Sub-caste \times Behavior	1	2.77	0.0963
Species \times Queen \times Sub-caste \times Behavior	1	0.0144	0.904
Colony within Species	2	5.90	0.0522

Table 4b. Results of fitting a multinomial logit model of the probability of *Acromyrmex* workers having oocytes falling into each of the three categories “non-yolky oocytes”, “yolky oocytes” and “resorbed oocytes”.

Source	df	Δ scaled deviance	p
Species	2	18.56	< 0.0001
Location (within or outside fungus garden)	2	18.13	0.0001
Sub-caste	2	12.94	0.0016
Species \times Location	2	0.259	0.879
Species \times Sub-caste	2	2.13	0.347
Location \times Sub-caste	2	3.11	0.211
Species \times Location \times Sub-caste	2	0.919	0.338
Colony replacing Species	6	7.17	0,305

resorbing ovarioles (Fig. 5). Since oocytes could fall into three categories (with yolk, without yolk, resorbed), the significance of this difference was tested using a GLM with multinomial logit error structure (Crawley, 1993). The dependent variable was the number of workers with oocytes in each of the three categories. Main effects were location of the worker (within or outside the fungus garden), species, and sub-caste. All interactions between main effects were also examined. After correction for over-dispersion in the data (Crawley, 1993), analysis was performed by fitting of the full model, followed by model simplification to yield the minimal adequate model. The significance of the removal of each term was assessed by comparing the associated change in scaled deviance and degrees of freedom with the chi-squared distribution. All the main effects, but none of the interactions, were retained in the minimal adequate model, showing that the proportion of workers with eggs in each of the three categories differed significantly between species (*Ac. echinator* workers had more yolky oocytes and resorbed

ovaries, and fewer non-yolky oocytes, than *Ac. octospinosus* workers), between sub-castes (LW had more yolky oocytes and resorbed ovaries, and fewer non-yolky oocytes, than SW) and depending on whether the worker was found inside or outside the fungus garden (interior workers had more yolky oocytes, and fewer non-yolky oocytes and resorbed ovaries than exterior workers). The effect of colony could not be examined directly by nesting it within species in such a model, so instead its significance was estimated by examining the change in scaled deviance caused by substituting colony for species in the minimum adequate model. This showed no significant difference between colonies in the proportion of workers with oocytes in the three different states that could not be explained by species differences. The full analysis is presented in table 4b.

We compared the length of yolky oocytes, irrespective of level of development, that were present in LW and SW. We fitted a GLM with oocyte length as a dependent variable, and species, colony nested within species, and sub-caste as explanatory variables. Only sub-caste was significant ($p < 0.0001$), while all other factors and interactions were non-significant ($p > 0.5$). This means that worker oocyte length does not differ between *Acromyrmex* species, but LW have significantly longer oocytes than SW. Thus, LW are not only more likely to have yolky oocytes than SW, but yolky oocytes are also on average larger in LW compared to SW.

Discussion

The loss of worker reproduction in attine ants

Our results show that most queenright workers in all four species studied do not lay reproductive eggs. We found no viable-looking eggs inside workers of *Ac. octospinosus*, *At. cephalotes*, and *At. sexdens*. Our results are thus consistent with the absence of worker sons among genotyped males from queenright *Ac. octospinosus* colonies (Boomsma et al., 1999) and we predict that worker sons will likewise be absent in queenright colonies of *At. sexdens* and *At. cephalotes*. In contrast, very low numbers of worker sons may occur in queenright colonies of *Ac. echinator*.

Acromyrmex workers, which can reproduce in orphaned colonies, show self-restraint in queenright colonies. Because *Acromyrmex* colonies may have thousands of workers and multiple fungus gardens, queens are unlikely to have the opportunity for queen policing. The observed reproductive self-restraint in *Acromyrmex* workers could be maintained by worker policing towards eggs or reproductive workers, provided that this worker policing is as effective as in honeybees (Ratnieks, 1988). Alternatively, worker self-restraint may be maintained by efficiency constraints related to worker morphology or worker-brood development time, which have recently been suggested to outweigh the relatedness advantage of worker sons versus queen sons in bumblebees (Duchateau et al., 2004). Detailed further laboratory studies will be needed to clarify which of these mechanisms apply in *Acromyrmex* leafcutter ants.

In contrast to *Acromyrmex*, *Atta* workers appear to be completely incapable of reproduction. Except for the queen retinue, workers do not develop their ovaries beyond the rudimentary stage present in newly-eclosed workers, and never produce eggs. As *Atta* and *Acromyrmex* are sister genera (Wetterer et al., 1998), we hypothesize that the evolution of worker sterility in *Atta* may have started with worker self-restraint as in *Acromyrmex*. Subsequently, larger colony size, increased queen-worker dimorphism, and a shorter life-span of orphaned colonies compared to *Acromyrmex*, combined with the costs of maintaining a reproductive apparatus in workers, may have made worker sterility adaptive in *Atta*. However, trophic functions of worker egg laying have been maintained in both genera of leafcutter ants, but in very different ways, so that we will focus the remaining part of the discussion on these aspects.

The maintenance and elaboration of trophic functions of worker egg-laying

A small subset of *Atta* workers, mostly SW and MW forming a retinue around the queen, lay many eggs in quick succession that are larger, more flaccid, and contain less yolk compared to queen-laid eggs. Bazire-Benazet (1957) observed that these eggs are fed to the queen. Physical contact with the queen may be the cue that induces *Atta* workers to develop trophic ovaries. The specialized morphology of the resulting eggs makes it highly unlikely that these eggs can ever develop. The occasional trophic egg layers that are found away from the queen may constitute a dynamic pool from which new queen nurses are recruited when needed. Alternatively, these workers may feed trophic eggs to the larvae. However, the rarity of trophic egg producing workers in queenless fungus gardens suggests that trophic eggs are not an important addition to the *Atta* larval diet. We found no evidence that *Atta* laying workers produce eggs through fusion of multiple oocytes, as reported by Bazire-Benazet (1957, 1970) for *Atta* queen nurses and founding queens. However, the trophic ovaries of *At. sexdens* and *At. cephalotes* workers look exactly as illustrated in Bazire-Benazet (1970). We frequently found multiple follicles containing large oocytes immediately adjacent to each other inside the ovariole, superficially suggesting fusion. However, these follicles were found to be separated by a ligament when the ovariole tunica was removed, without visible outgrowths of the oocytes into adjacent follicles. Also, contrary to the expectation if *Atta* worker eggs are produced through oocyte fusion, ready-to-lay eggs were not larger in workers of *Atta* than in *Acromyrmex* (Fig. 3). We thus remain unconvinced of the occurrence of oocyte fusion in *Atta* workers, although the phenomenon is well documented in founding queens (Bazire-Benazet 1957).

In *Acromyrmex*, many workers lay trophic eggs that contain less yolk than queen-laid *Acromyrmex* eggs, but more than *Atta* worker-laid eggs. In all studied aspects, *Acromyrmex* worker ovaries were similar to the ovaries of workers of *Myrmica rubra*, a non-fungus-growing myrmicine ant, as described by Minkenbergh and Petit (1985). Indi-

vidual *Acromyrmex* workers can lay only one egg per ovary at a time and must have a refractory period of several days between successive ovulations from the same ovary. Laying *Acromyrmex* workers appear to lay multiple eggs between eclosion and middle age. We never found trophic eggs lying among the brood, suggesting that they are fed directly to the larvae. The high frequencies of workers producing eggs among *Acromyrmex* workers caring for the larvae and pupae strongly suggest that their eggs are fed to the larvae, and possibly to the queen. Trophic worker-laid eggs may thus be an important part of the *Acromyrmex* larval diet, in addition to the fungal hyphae and gongylidia that are the staple larval food of all attine ants.

In *Acromyrmex*, large workers contain mature trophic eggs far more frequently than small workers, indicating that large workers are the main source of trophic eggs. The equal development of *Acromyrmex* worker ovaries in queenright and queenless fungus gardens suggests that proximity to the queen does not affect worker ovary development. This agrees with our conclusion that *Acromyrmex* worker-laid eggs are mainly destined to be eaten by larvae, as larvae are found in all fungus gardens. More workers produce eggs in *Ac. echinator* than in *Ac. octospinosus*, suggesting that trophic eggs are a more important part of the larval diet in *Ac. echinator* than in *Ac. octospinosus*. The higher frequency of trophic eggs inside worker ovaries of *Ac. echinator* than of *Ac. octospinosus* is also found when small, non-reproducing colonies are used (M.B. Dijkstra, personal observation). Thus the production of sexuals by the study colonies of *Ac. echinator*, but not of *Ac. octospinosus* (See Table 1), cannot explain the observed pattern. Some (0.8%) of the dissected *Ac. echinator* workers, but no *Ac. octospinosus* workers, had a normal-looking egg inside one of their ovaries. However, we may have underestimated the frequency of reproductive workers in *Acromyrmex* colonies, if intermediate-type eggs can also occasionally develop. Genotyping of developing eggs is necessary to solve this question. As *Acromyrmex* trophic and reproductive eggs appear to have similar morphologies apart from yolk density, individual *Acromyrmex* workers may shift to producing reproductive eggs in orphaned colonies by increased metabolism of the fat body and increased yolk uptake into the oocytes. *Acromyrmex* and *Atta* workers with ready-to-lay eggs that are kept in isolation do not spontaneously lay eggs, suggesting that workers can control ovulation and lay eggs only when needed. A larval- or queen-produced cue or signal may be necessary to induce ovulation in leafcutter ant workers.

In all four species studied, the older workers that work outside the fungus gardens tend to resorb their ovaries, which leaves them permanently unable to lay any eggs. Assuming that the main function of trophic eggs in *Acromyrmex* is larval feeding, the abortion of this function at a later age when nursing tasks are replaced by foraging tasks seems a logical development, because cessation of ovary maintenance may well enhance worker longevity or colony-level productivity at that stage.

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