

Degeneration of sperm reservoir and the loss of mating ability in worker ants

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Abstract Workers never mate in the large majority of ants, and they have usually lost the spermatheca, an organ specialized for long-term storage of sperm. Such ‘non-sexual’ workers are restricted to laying unfertilized eggs that give rise to males, and they cannot compete with the queens for the production of female offspring. In sharp contrast, workers in 200–300 species from phylogenetically basal subfamilies can reproduce sexually (‘gamergates’) because they retain a functional spermatheca like the queens. Importantly, ‘non-sexual’ workers in closely related species have a vestigial spermatheca. In this study, we compared the reservoir epithelium of ‘sexual’ workers to that of congeneric queens and ‘non-sexual’ workers using 21 species of Amblyoponinae, Ponerinae and Ectatomminae. We show that a pronounced thickening of the epithelium near the opening of the sperm duct is strictly

associated with sexual reproduction in both castes. This is unlike ‘non-sexual’ workers in which this epithelium is always very thin, with few organelles; but all other structures remain intact. We discuss this evolutionary degeneration of the spermatheca and how it relates to behavioural or physiological modifications linked to mating. Our results help understand the loss of sexual reproduction by ant workers, a critical step in the extreme specialization of their phenotype.

Keywords Spermatheca · Queen worker dimorphism · Caste · Reproductive conflict · Sperm storage

Introduction

The occurrence of many non-breeding individuals is fundamental to the organization of insect societies. In ants, some social bees and wasps, breeders and helpers are morphologically distinct castes, i.e. queens and workers. The latter generally cannot mate and are thus restricted to producing males from unfertilized eggs. The potential for reproductive conflict is consequently reduced since queens and workers compete only for the production of haploid male offspring (Ratnieks 1988). Hence, conflicts are restricted to worker policing in the presence of a queen (e.g. workers destroy the male eggs laid by virgin nestmate workers if these eggs are less related to them compared to the queen’s male eggs; Ratnieks et al. 2006). This is unlike social insects in which both breeders and helpers have the ability to mate, e.g. some ants that have lost the queen caste, and bees and wasps without morphological castes (Heinze et al. 1994; Monnin and Peeters 2008; Ross and Matthews 1991). Nestmates thus compete for both haploid and diploid reproduction, and aggression-based hierarchies

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regulate mating and oviposition (e.g. the ant *Dinoponera quadriceps* Monnin and Peeters 1998; the bee *Exoneura nigrescens* Langer et al. 2004; the wasp *Polistes dominulus* Reeve 1991). The loss of mating ability in helpers is an important evolutionary step towards a reduction of reproductive conflicts because a division of labour based strictly on caste morphology improves colonial efficiency (Bourke 1999).

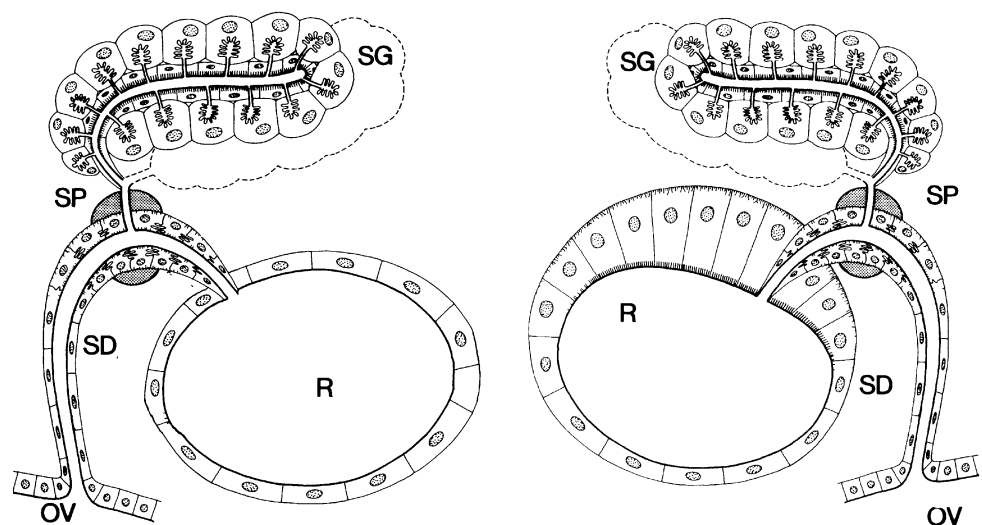
Phenotypic differentiation of female adults reaches an extreme in various ants that have colonies with 10^5 or more workers (e.g. leaf-cutter *Atta*, *Carebara*, army ants *Dorylus*; Hölldobler and Wilson 1990). Queens in such species are extremely fertile and very long-lived, while the workers cannot lay eggs. However, many degrees of queen–worker dimorphism exist among the ants. The evolution of the worker phenotype can be envisaged in three major steps. Firstly, workers became wingless as an adaptation to life on the ground. This step irreversibly compromised their option to leave natal colonies and start a new colony individually, as they lack wings to disperse and wing muscles as energetic reserves (Peeters and Ito 2001). Nevertheless, workers at this evolutionary stage can still mate and compete with the queens for breeding, as seen in a few hundred phylogenetically basal species (Peeters 1991; Peeters and Ito 2001). The second step is loss of the ability to mate and store sperm, which is typical of workers in most ants. Such workers have ovaries but lack a spermatheca. Only relatively few species exhibit the third evolutionary step, i.e. complete loss of ovaries (Bourke 1988). Here, we focus on the second step—workers can no longer fertilize their eggs, hence only the queen(s) can produce female offspring—and the importance of the spermatheca.

Females in the social Hymenoptera mate only once in their lives, and thus, long-term storage of sperm is crucial.

The spermatheca in ants consists of a spherical reservoir connected to the oviduct with a sperm duct (Fig. 1); through this duct, sperm enters the reservoir during mating and exits again for fertilization of eggs (Wheeler and Krutzsch 1994; Allard et al. 2005; Oppelt and Heinze 2007). The spermatheca is a specialized organ providing a chemically stable environment in which sperm remains viable for years. Investigations of bumblebees, honeybees and ants indicate that the reservoir epithelium is likely to have an excretory function, withdrawing ions and sperm waste products from the lumen (Wheeler and Krutzsch 1994; Koeniger 1970; Dallai 1975). The hilar epithelium, near the opening of the sperm duct (Fig. 1), was identified to have an important transport function in the long-lived queens of the Myrmicinae ant *Crematogaster opuntiae* (Wheeler and Krutzsch 1994). Gobin et al. (2006) confirmed the importance of the hilar epithelium in the spermatheca of queens from 19 species in the basal subfamily Ponerinae.

In the majority of ants, the spermatheca is completely absent in the worker caste (Hölldobler and Wilson 1990). The permanent loss of this essential organ is a significant component of caste dimorphism. If we assume that ‘non-sexual’ workers evolved gradually, intermediate stages are most likely to be found in species exhibiting weak queen–worker divergence. In basal subfamilies, the castes often differ little in external size as well as number of ovarioles (Peeters 1993). In 200–300 species belonging to several independently evolved genera, workers are able to mate and lay fertilized eggs. In such species, ‘gamergates’ (mated and breeding workers) can reproduce together with queens, or gamergates can replace queens permanently (Peeters and Ito 2001). Importantly, workers in closely related species or genera never mate [i.e. the queen(s) monopolize sexual reproduction], but they have retained a visually identifiable

Fig. 1 General organization of the spermatheca in ants: *left* workers of Q species lacking worker mating; *right* workers of G and G+Q species with worker mating. Workers that can mate have a sperm reservoir (R) with a columnar hilar epithelium, similar to the ant queens. ‘Non-sexual’ workers lack this specialized epithelium. Other features are similar in both worker types: The paired spermatheca gland (SG) and sperm reservoir (R) are attached to the common oviduct (OV) with a sperm duct (SD)



spermatheca with a simplified reservoir epithelium (Gobin et al. 2006; Ito and Ohkawara 1994).

Here, we present the first comparative study of the evolutionary degeneration of the spermatheca and loss of mating ability in ant workers. We investigated 21 phylogenetically basal species that exhibit three distinct reproductive strategies: queens and no gamergates, queens and gamergates, gamergates only. Histological techniques were used to compare spermatheca morphology in ‘sexual’ workers and queens. We discuss whether it is possible to re-evolve ‘sexual’ workers in basal ants.

Materials and methods

We studied the ultrastructure of the spermatheca reservoir and glands in 21 species of ants. Most of these belong to the subfamily Ponerinae (16 species in nine genera), but two other subfamilies that have gamergates were also included (Table 1). With the notable exception of *Pachycondyla*, all genera investigated here appear to be monophyletic (Bolton 2003; Brady et al. 2006; Moreau et al.

2006), and this increases the relevance of comparing congeneric species with or without gamergates. We did not aim for a formal comparative analysis (e.g. independent contrasts), but included a broad sample of species and genera to obtain a better picture of natural variability. Only two genera with known gamergate reproduction were unavailable for inclusion in our sample: *Streblognathus* (Ponerinae) and *Rhytidoponera* (Ectatomminae; Cuvillier-Hot et al. 2004; Ward 1981).

Only species with known reproductive strategies—from unpublished data or the literature—were included (Table 1). Seven species exhibiting both queen and gamergate reproduction (Q+G; e.g. *Pachycondyla astuta*) allowed for direct comparisons of breeders belonging to different castes. Gamergates do not exist in another nine species (Q), and some of these were selected because of different degrees of queen–worker dimorphism (e.g. *Pachycondyla obscuricornis* and *Paltothyreus tarsatus*) or to compare with congeners exhibiting Q+G. Another five species have gamergates only (G), and *Leptogenys peuqueti* (G) was compared with *Leptogenys kraepelini* (Q). Thus, in both *Leptogenys* and *Pachycondyla*, ‘sexual’ workers could be

Table 1 Ant species in which the spermatheca was studied histologically

| Subfamily | Species | Caste of breeders | <i>N</i> queen | <i>N</i> worker | Country | Authority for reproductive biology |
|--|--|-------------------|----------------|-----------------|---------------------------------|---|
| Amblyoponinae | <i>Amblyopone reclinata</i> | G | / | 5 | ID | Ito 1993 |
| Ectatomminae | <i>Gnamptogenys bicolor</i> | Q+G | 2 | 3 | ID | Ito unpublished (<i>n</i> =13) |
| | <i>Gnamptogenys costata</i> | Q | 2 | 3 | ID, MY | Ito unpublished (<i>n</i> =5) |
| | <i>Gnamptogenys menadensis</i> | Q+G | 5 | 7 | ID, MY | Gobin et al. 1998, 2001; Gobin unpublished (<i>n</i> =61) |
| | <i>Gnamptogenys moelleri</i> | Q+G | 3 | 2 | BR | Gobin unpublished (<i>n</i> =1); Blatrix personal communication (<i>n</i> =2) |
| Ponerinae | <i>Anochetus</i> sp. | Q | 1 | 2 | ID | Ito unpublished (<i>n</i> =2) |
| | <i>Diacamma</i> sp. | G | / | 3 | ID | Peeters et al. 1992 |
| | <i>Dinoponera quadriceps</i> | G | / | 5 | BR | Monnin and Peeters 1998 |
| | <i>Harpegnathos saltator</i> | Q+G | 4 | 4 | IN | Peeters and Hölldobler 1995 |
| | <i>Leptogenys kraepelini</i> | Q | 4 | 3 | ID | Peeters and Ito 2001; Ito 1997 |
| | <i>Leptogenys peuqueti</i> | G | / | 5 | ID | Ito 1997 |
| | <i>Leptogenys</i> sp. 25 | G | / | 5 | ID | Ito unpublished (<i>n</i> =2) |
| | <i>Leptogenys parvula</i> | Q | 1 | 2 | ID | Ito unpublished (<i>n</i> =8), Peeters and Ito 2001 |
| | <i>Myopias</i> sp. 8 | Q | 4 | 3 | ID | Ito unpublished (<i>n</i> =3) |
| | <i>Myopias emeryi</i> | Q | 3 | 1 | MY | Ito, unpublished (<i>n</i> =22) |
| | <i>Odontomachus rixosus</i> | Q | 6 | 5 | ID | Ito et al. 1996 |
| | <i>Pachycondyla obscuricornis</i> | Q | 3 | 6 | CR | Oliveira and Hölldobler 1991; Gobin et al. 2003 |
| | <i>Pachycondyla (Ectomomyrmex) astuta</i> | Q+G | 1 | 2 | ID | Ito unpublished (<i>n</i> =14) |
| | <i>Pachycondyla (Bothroponera) sp. 10</i> | Q+G | 1 | 4 | MY | Ito unpublished (<i>n</i> =4) |
| | <i>Pachycondyla (Paltothyreus) tarsata</i> | Q | 3 | 4 | ZA | Villet et al. 1989; Braun et al. 1994 |
| <i>Platythyrea</i> sp.1 (near <i>parallela</i>) | Q+G | 2 | 1 | ID | Ito unpublished (<i>n</i> =12) | |

Queen and worker castes were compared (*N* is sample size), taking into account whether workers are ‘sexual’ or not. Voucher specimens of unidentified species were deposited in the Museum of the Bogor Botanical Gardens, Indonesia. For unpublished data on reproductive biology, *n*=examined colonies

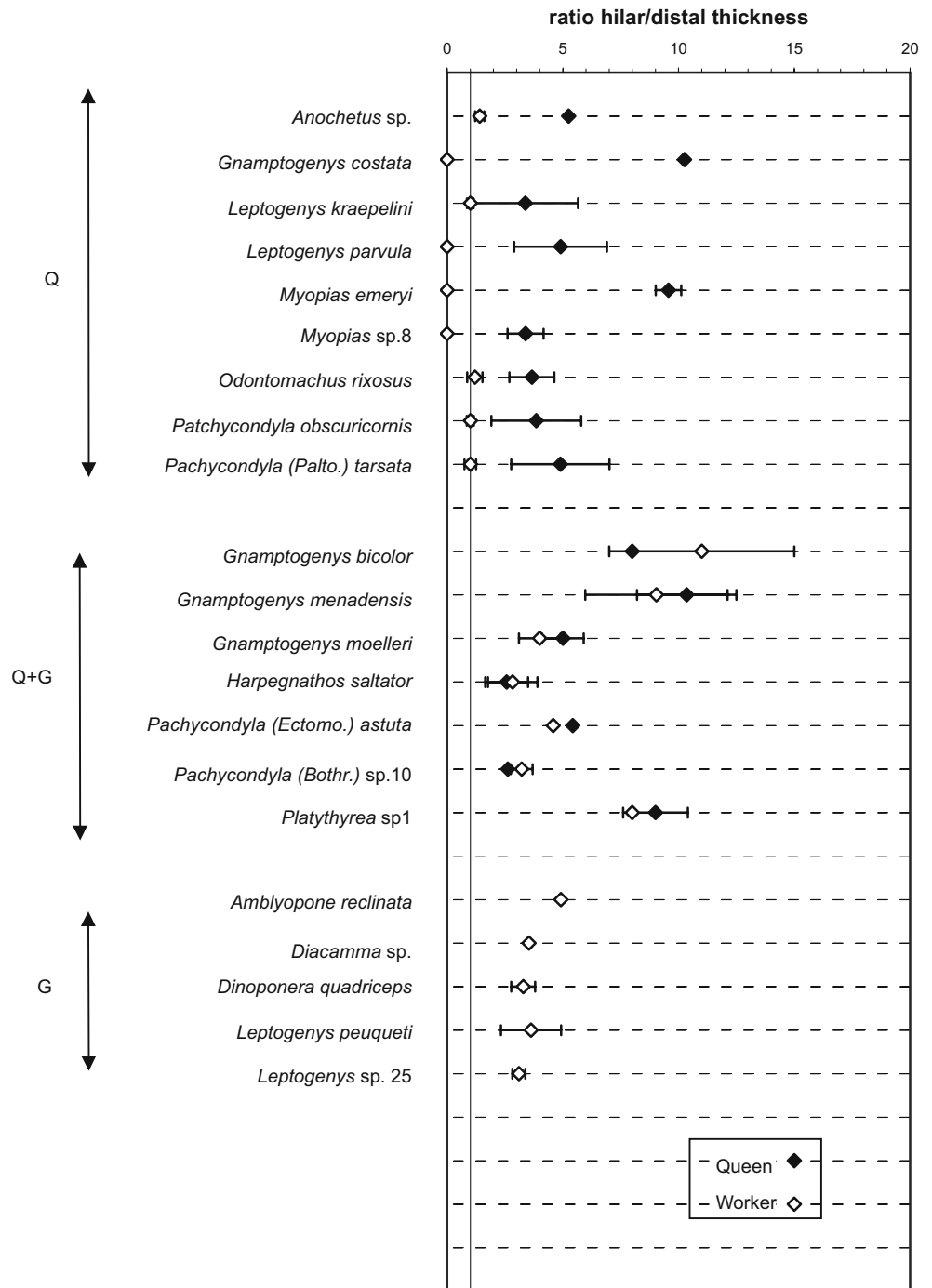
Q No gamergates; G no queens; Q+G queens and workers reproduce; “/” caste absent; BR Brazil; CR Costa Rica; ID Indonesia; IN India; MY Malaysia; ZA South Africa

compared with workers that never mate. Observed differences were then compared to *Odontomachus* and *Myopias*, genera in which worker mating is completely unknown.

We studied a few specimens in each caste (Table 1) and included virgin and inseminated individuals in four species to verify a mating effect on reservoir morphology. Spermathecae of queens and workers were dissected in Ringer’s solution at $\times 10$ magnification. Spermathecal

tissues were fixed in 2% glutaraldehyde in sodium cacodylate buffer, postfixed in 2% osmium tetroxide and dehydrated in a graded acetone series before embedding in Araldite. Serial 1 μm semithin sections for light microscopy were stained with methylene blue. A number of samples for each genus were examined at a subcellular level. Thin sections (70 nm) were double-stained with uranyl acetate and lead citrate for transmission electron microscopy (see Gobin et al. 2006 for details).

Fig. 2 Ratio of the thickness of hilar and distal epithelia of spermatheca reservoirs in queens (black) and workers (white) of species from Table 1. Species with similar reproductive strategy are grouped together: *Q* no gamergates, *G* no queens, *Q+G* queens and workers reproduce). Standard deviation bars are indicated when three or more individuals were studied. A zero value indicates complete absence of spermatheca



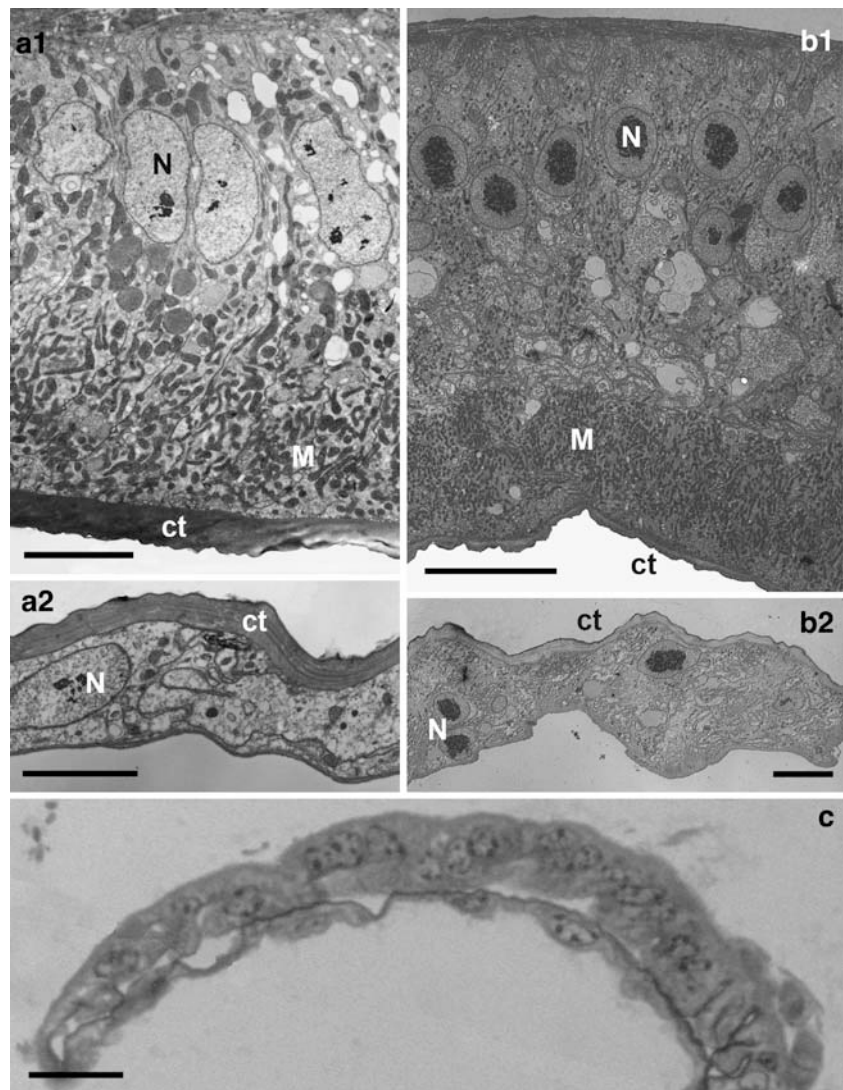
All measurements were performed on an Olympus BX51 at $\times 200$ – 400 magnification with a Camedia camera and DP Soft software. To measure the thickness of reservoir epithelia, we identified the best section of the hilar region around the opening of the sperm duct and measured the width of the hilar epithelium as well as that of the distal epithelium opposite (see Fig. 1). We calculated the ratio of hilar over distal epithelium as a representative value of hilar epithelium development and plotted the mean ratios and standard deviations in the case of multiple specimens.

Results

Ant workers that are able to mate and lay fertilized eggs exhibit a conspicuous difference in the walls of their sperm

reservoir (Fig. 1) compared to related species with ‘non-sexual’ workers. In all gamergate species, workers have the hilar wall (near the opening of the sperm duct) three to ten times thicker than the distal wall (Fig. 2). Similar thickening of the hilar wall was also found in all queens investigated. In contrast, workers that never mate (Q species) have a ratio close to 1 (Fig. 2), reflecting a thin epithelium in both hilar and distal regions, and this clearly separates them from conspecific queens. However, musculature, glands and ducts associated with the spermatheca are present in all workers and show no qualitative variation associated with mating ability. We found no difference in epithelium thickness between virgin and mated individuals when comparing queens (*Odontomachus rixosus*: three mated vs. three virgin examined) or workers (*Gnamptogenys menadensis* three vs. four; *L. pequeti* three vs. two; *Amblyopone reclinata* four vs. one).

Fig. 3 Composite electron micrographs of the spermatheca reservoir: gamergate of *G. moelleri* (**a** 1 hilar epithelium, 2 distal epithelium; both scale bars=5 μm) and a queen of *P. tarsata* (**b** 1 hilar epithelium; 2 distal epithelium; both scale bars=10 μm). Note the abundance of cell organelles in the hilar epithelium. **c** Light microscopic section of the spermatheca reservoir of a non-sexual worker of *P. tarsata*. Both hilar and distal epithelia are flat and have few organelles (scale bar=100 μm). *ct* cuticle, *M* mitochondria, *N* nucleus



Since the spermatheca wall consists of a single cell layer with a slender cuticle lining the lumen (Fig. 3), the variations in thickness depend entirely on intracellular specialization. The highly columnar cells of the hilar epithelium of ‘sexual’ workers have similar ultrastructural organization as in queens (Fig. 3a, b), e.g. abundant mitochondria and glycogen, weakly developed endoplasmic reticulum and Golgi apparatus (Fig. 4b, c). Distinct membrane foldings on both the lumen and haemolymph sides facilitate transcellular transport. In contrast, the hilar epithelium of ‘non-sexual’ workers has a flattened, cuboidal appearance (Fig. 3c), few organelles and few membrane invaginations (Fig. 4a), indicating the absence of significant metabolic activity.

In individual species where both workers and queens reproduce sexually (Q+G), both castes have similar absolute widths of hilar and distal epithelia (Fig. 2). Further interspecific comparisons across the three subfamilies confirmed that a thickened hilar epithelium is always characteristic of sexual reproduction. Congeneric species that differ with respect to the existence of gamergates, e.g. *L. kraepelini* and *L. peuqueti* or *P. obscuricornis* and *P. astuta*, exhibit thickened hilar epithelia of workers only when gamergates exist. In four of the Q species investigated, the workers lacked a spermatheca altogether (Fig. 2).

Discussion

Hymenopteran females are sexually receptive for only a brief period at the beginning of their adult life, and they

will not mate again. Long-term sperm storage is thus a crucial adaptation in social insects with perennial colonies. Using a sample of 30 phylogenetically basal ant species characterized by limited queen–worker dimorphism, Gobin et al. (2006) showed that the ability of queens to store sperm is associated with the specialization of the hilar epithelium in the spermatheca reservoir. Queens always have a thick epithelium, whereas ‘non-sexual’ workers have a thin one. In this study, we show that in related species where workers mate regularly, their reservoir morphology is strikingly similar to the queens’. Abundant mitochondria and other organelles together with membrane foldings in the columnar epithelium of ‘sexual’ workers hint at an excretory function, as in queens. This contrasts with the thin hilar epithelium in ‘non-sexual’ workers (Q species) lacking microvilli and with only few mitochondria. This was found in all Q species, irrespective of whether they belong to genera that have species with worker mating (i.e. *Pachycondyla* and *Leptogenys*) or genera without G species (i.e. *Odontomachus*).

We documented an all-or-none modification in the spermatheca reservoir of workers according to species-specific mating ability. The degeneration of a single tissue, the hilar epithelium, occurs in ‘non-sexual’ workers from phylogenetically separate genera of Ponerinae as well as in two other subfamilies. Such a uniform pattern underlines the importance of the columnar epithelium for keeping sperm viable for years. Since columnar epithelium with abundant organelles has a cost, which is multiplied by the number of workers produced in each colony, it may be expected that, in species where queens are selected to monopolize sexual

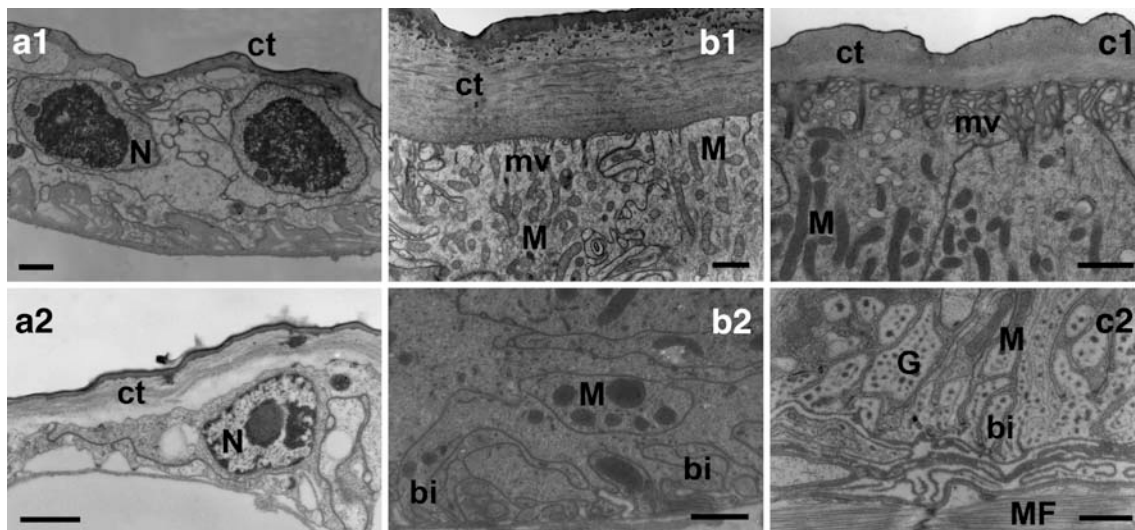


Fig. 4 Electron micrographs of the spermatheca reservoir in workers (a 1, 2), gamergates (b 1, 2) and queens (c 1, 2). a 1 *P. tarsatus* worker, a 2 *P. apicalis* worker, b 1 *D. quadriceps* gamergate, b 2 *H. saltator* gamergate, c 1 *P. tarsatus* queen, c 2 *O. rixosus* queen. Scale

bar in all figures=1 μ m. bi Basal invaginations, ct cuticle, G glycogen, M mitochondria, MF muscle fibres, mv microvilli, N nucleus

reproduction, workers have a reservoir with a degenerate epithelium. However, it is striking that other parts of the spermatheca, i.e. ducts, muscles and accessory glands, remain intact in ‘non-sexual’ workers belonging to basal subfamilies (Gobin et al. 2006). These structures are linked to sperm release and reactivation (Koeniger 1970; Wheeler and Krutzsch 1994; Schoeters and Billen 2000), and they clearly also have a cost. Considering our sample, ‘non-sexual’ workers in only four species completely lacked a vestigial spermatheca (Fig. 2); this includes *Myopias* in which G species are completely unknown.

What is the significance of retaining a vestigial spermatheca in ‘non-sexual’ workers of many basal ants? This contrasts with its complete absence in workers of all higher ants (Hölldobler and Wilson 1990). It is hardly surprising that a vestigial spermatheca eventually disappears because maintaining such a non-functional organ clearly requires energy. Its retention in basal ants is consistent with the limited divergence between queen and worker castes. Caste differences in the phenotypic expression of a single trait generally results from switching off one or a few genes during larval development (Evans and Wheeler 1999). However, spermatheca genes that are not expressed in workers must remain functional in queens (Abouheif and Wray 2002 discussed this in the context of halting wing development in worker larvae). It remains an open question whether the degeneration of the reservoir epithelium is reversible and whether ‘non-sexual’ workers in various Ponerinae can regain the ability to store sperm. Gobin et al. (2006) showed that most tissues in their vestigial spermathecae are intact, and thus, such reverse evolution may occur readily.

The majority of species in Amblyoponinae and Ponerinae lack gamergates, and the latter have a scattered occurrence, suggesting that sexual reproduction by workers is a derived condition, possibly linked to the adaptive benefits of secondary reproductives, e.g. increased colony lifespan and resource inheritance (Peeters and Ito 2001; Monnin and Peeters 2008). Our histological results revealed no difference between the spermathecae of ‘sexual’ workers and conspecific queens. In basal species where gamergate reproduction becomes selected for, ‘non-sexual’ workers may express specific genes to produce more mitochondria and other organelles, thus regaining a columnar epithelium. The current lack of species-level phylogenies in genera with scattered Q+G or G species prevents testing these ideas.

In gamergate species, workers copulate with foreign males inside or near the entrance of their nests (Peeters 1991). Only the dominant worker(s) are sexually attractive (e.g. Monnin and Peeters 1998; Gobin et al. 2001), and pheromones are probably involved. In several Q species from basal subfamilies, the winged gynes do not engage in

nuptial flights and instead exhibit sexual calling on the ground (references in Peeters and Ito 2001). In various Q+G species such as *Rhytidoponera confusa*, both winged gynes and workers mate outside the nest entrances, albeit in different colonies (Ward 1981). When worker mating is selected against, we can only speculate whether behavioural or physiological modifications occur before or after the loss of columnar epithelium in the sperm reservoir.

Gamergates do not occur in any higher ants. Once workers evolve into obligate helpers, they cannot produce female offspring, and intracolony reproductive conflicts become confined to the production of males (Ratnieks 1988; Ratnieks et al. 2006). The loss of the specialized reservoir epithelium in the workers of many basal ants appears to be an important step in the evolution of obligate helpers, but this loss appears reversible in basal species where gamergate reproduction becomes selected for.

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