

Differentiation of the reproductive tract between dominant and subordinate workers in the Japanese queenless ant *Diacamma* sp.

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Keywords:

bursa copulatrix, *Diacamma*, gemmae, queenless ants, spermatheca

Accepted for publication:

5 July 2005

Abstract

Allard, D., Ito, F., Gobin, B., Tsuji, K. and Billen, J. 2005. Differentiation of the reproductive tract between dominant and subordinate workers in the Japanese queenless ant *Diacamma* sp. — *Acta Zoologica* (Stockholm) 86: 159–166

In queenless ants, gamergates (mated egg-laying workers) fulfil the reproductive task normally reserved for the queen. Every worker is a potential gamergate, thus we expect pronounced conflicts over sexual reproduction within their colonies. In the queenless ant genus *Diacamma*, gamergates inhibit nest mates from mating by aggressively removing ('mutilating') a pair of small appendages on the thorax, termed gemmae, shortly after eclosion. Dissection and serial sectioning of the reproductive tracts of both mutilated and unmutilated individuals of *Diacamma* sp. from Japan at different ages revealed that mutilation inhibits the development of the bursa copulatrix and the spermatheca, two structures fundamental for sexual reproduction. The precursor of the bursa copulatrix develops into a fully functional structure in unmutilated individuals, whereas it degenerates irreversibly in mutilated callows. Experimental manipulations showed that the removal of the gemmae is not the sole factor regulating this development. The spermathecal epithelium and accessory spermathecal gland of unmutilated individuals are thicker than that of mutilated individuals, indicating a higher degree of activity in the former. Mutilated females are therefore left incapable of copulating and less competent for long-time sperm storage.

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Introduction

Despite the harmonious façade of a social insect colony, many aspects of social life are shaped by conflict. Conflict among kin arises from differences in relatedness with sexual offspring and/or in productivity (Bourke and Franks 1995). For social Hymenoptera, numerous types of conflict have been predicted by the kin conflict theory and documented over the past decades (for an extensive review refer to Bourke and Franks 1995); a spectacular example is the lethal fighting between newly emerged queens in colonies of the honey bee *Apis mellifera* (Visscher 1993). Since a female is more related to her own offspring than to her sister's, each queen should value herself more highly than her sisters to replace their mother at the head of the colony.

An analogous phenomenon can be observed in queenless ants. Several ant genera, including *Diacamma*, have lost the queen caste. The reproductive tasks are fulfilled by one or several gamergates, i.e. mated workers, and this evolutionary replacement of queens by workers is possible because workers in these genera have retained a spermatheca and ovaries (Peeters 1991). Gamergates can lay both unfertilized eggs, which develop into males, and fertilized eggs which develop into females, according to the principle of haplodiploidy. On the other hand, subordinate workers can only lay unfertilized, male-destined eggs in the absence of the gamergate. In this social system, one might expect a conflict among workers over who will become a gamergate (a mated worker) and increase its personal fitness. In fact, the regulation of mating activity in queenless ants ranges

from the apparent lack of regulation in species with multiple workers who mate with foreign males visiting the nest [*Ophthalmopone berthoudi* (Peeters and Crewe 1985), *Harpegnathos saltator* (Peeters and Hölldobler 1995)], to aggressive interactions that inhibit mating in subordinates [*Pachycondyla sublaevis* (Ito and Higashi 1991), *Pachycondyla* (= *Bothroponera*) sp. (Ito 1993), *Dinoponera quadriceps* (Monnin and Peeters 1998) and *Gnamptogenys menadensis* (Gobin et al. 2001a)]. In the latter group, the proximate mechanisms linking dominance status to mating ability are largely unknown. Existing studies on regulation of reproductive traits in ants generally focus on the evolutionary outcome of conflicts rather than on the mechanisms. By observations and experimental manipulations (selective control of one potential signal at a time), it is possible to assess which factor causes reproductive inhibition in subordinate ants. Aggression, egg cannibalism and pheromonal signals are generally invoked as the causal factors in reproductive inhibition (Passera 1980; Hölldobler and Wilson 1983). It remains largely unknown how aggression or pheromonal signals result physiologically in the absence of egg-laying and the lack of insemination.

In *Diacamma* species, workers are inhibited from mating by the single gamergate through a peculiar behaviour: the mutilation of the gemmae (Fukumoto et al. 1989; Peeters and Higashi 1989; Sommer et al. 1993). Every worker ecloses with a pair of these tiny bladder-like appendages of the mesothorax. Shortly after eclosion, the young worker is immobilized by nest mates and the gemmae are aggressively bitten off by the gamergate (which is the only individual in the colony that retains her gemmae). This mutilation affects the mating ability of the callow: mutilated workers never mate. When the gamergate dies, the next worker to eclose retains her gemmae and mutilates all callows eclosing after her. After about two weeks, she will start performing sexual calling behaviour in and outside the nest to attract a male. Copulation will start outside the nest, but the female soon joins her nest mates in the safety of the nest, with her male partner still attached to her abdomen. The male is usually quickly killed and dismembered by the female and her nest mates but his abdomen can remain attached to the female's genital opening for a number of hours (Fukumoto et al. 1989). After being freed from the male, the young gamergate starts laying fertilized eggs and expanding the colony.

The present study aims to clarify the proximate mechanisms linking mutilation status to mating ability in *Diacamma*. What exactly hinders mutilated workers from mating? Upon discovery of glandular tissue in the *Diacamma* gemmae, Peeters and Billen (1991) hypothesized that the gemmae might release a sexual pheromone. Mutilated females would then be incapable of attracting a male. However, Nakata et al. (1998) identified the metatibial gland in Japanese *Diacamma* hind legs as the main source of sexual attractants and excluded the gemmae from this function. The discovery

of a nerve fibre connecting the gemmae to the central nervous system and the degeneration of these neuronal afferents upon mutilation (Gronenberg and Peeters 1993) suggested a coupling with physiological (and possibly behavioural) processes.

In this study we compare the development of genital structures essential for sexual activity between mutilated and unmutilated virgin workers in *Diacamma* sp. from Japan, previously referred to as *Diacamma rugosum* (Le Guillou) by Fukumoto et al. (1989). We focus on the bursa copulatrix and the spermatheca, two structures with known importance for sexual reproduction. In *Diacamma* sp., the bursa copulatrix is used to establish the firm and safe connection with the male genitals during copulation (Allard et al. 2002). In addition to dissection and histological sectioning of this organ in both mutilated and unmutilated females, we perform a series of experimental treatments to identify the factor responsible for the differential development of this structure. The aggression from nest mates and a signal from the reproductive individual are commonly invoked as inhibiting factors in social insects. The factor 'gemmae' is unique to the genus *Diacamma* and still puzzles science as to its origin and function. We attempt to separate the effects of, on one hand, the aggression experienced by the callow during the mutilation process (biting, forced immobilization, pulling), closely associated with the presence of the gamergate and, on the other hand, the pure removal of the gemmae. The ant spermatheca consists of a reservoir, connected to the oviduct by the spermathecal duct, and a paired accessory gland, connected to the spermathecal duct (Wheeler and Krutzsch 1994). The reservoir stores the sperm received from the male and keeps it viable for several months/years. The epithelium of the sperm reservoir has a transporting function, keeping the environment of the spermathecal lumen optimal for the stored sperm. The secretion of the spermathecal gland of social insects apparently activates the sperm released into the spermathecal duct for fertilization of eggs (Koeniger 1970; Wheeler and Krutzsch 1994). Ito and Ohkawara (1994) found no difference in the size of the spermathecal reservoir between gamergates and uninseminated workers for nine genera of ponerine ants, including *Diacamma*. We here study the spermatheca in more detail and measure the thickness of the reservoir epithelium and the diameter of the accessory gland.

Materials and methods

Collection and laboratory rearing

Fourteen colonies of *Diacamma* sp. were excavated on Okinawa Island, Japan, during April 2000 and September 2002, and transferred to Belgium. In the laboratory, colonies were kept in artificial nests consisting of a plaster foraging arena (5 × 20 × 20 cm) with a small sunken nest chamber (0.5 × 5 × 15 cm) covered by a glass plate and red plastic

Table 1 Characteristics and numbers of the different types of *Diacamma* sp. workers reared in the laboratory

	Possession (+) or lack (-) of gemmae	Presence (+) or absence (-) of gamergate	N	Bursa copulatrix		
				Developed	Not developed	Not examined
Natural conditions						
Unmutilated	+	-	(See Table 2)			
Mutilated	-	+				
Experimental conditions						
Type A	- (artificially)	-	10	6	4	0
Type B	+ (coated)	+	12	0	2	10
Type C	- (naturally)	+	17	0	16	1
Type D	+ (coated)	-	19	18	0	1

foil. The nests were kept at a constant temperature (25 °C) and constant relative humidity (75%), and were exposed to a regular day/night cycle (12 h/12 h). The ants were fed with mealworms and small crickets every other day, and supplied with water in a glass tube sealed with a cotton plug.

Rearing of mutilated and unmutilated females

We reared the two types of workers, mutilated and unmutilated, and dissected them at different ages after eclosion (1, 5, 10, 15 and 30 days), with 10 days being the age at which unmutilated virgins are ready to mate (Fukumoto *et al.* 1989).

To rear unmutilated workers, we created 16 experimental groups each composed of mutilated workers, marked with a dot of paint on the abdomen, and a single cocoon. The nests were checked daily for newly eclosed callows. As soon as a callow had emerged from its cocoon it was marked with paint. These callows were not mutilated because they were reared in the presence of already mutilated workers. Every colony contained only one unmutilated callow at a time.

To rear mutilated workers of known age, we created eight experimental groups consisting of a gamergate, mutilated marked workers, and a few cocoons. The nests were checked daily for freshly eclosed callows, that bear no paint. These callows were naturally mutilated in their colony. They were each marked with an individual colour-code and placed back into their nest.

Each experimental group consisted of individuals from the same stock colony. The size of the groups ranged from five to approximately 30 individuals, depending on the number of workers available in the stock colonies.

The callows were taken from their nest for dissection when they reached the desired age (1, 5, 10, 15 or 30 days, randomly assigned to each individual). We dissected a total of 27 unmutilated workers and 32 mutilated workers (Table 2).

Experimental manipulation and development of the bursa copulatrix

To investigate the importance of the gemmae vs. the factors aggression and presence of the gamergate on the development of the bursa copulatrix, we reared four additional types of females (see Table 1). Young unmutilated females were obtained as described in the previous section, and subjected to four different experimental treatments on the day of their eclosion. To check if the sole removal of the gemmae is responsible for the degeneration of the bursa, we artificially mutilated callows by removing both gemmae with a fine dissecting tool right after eclosion, after which the workers were placed back in their nest. The 'type A' females obtained this way were thus reared in the absence of a gamergate, hence experiencing no aggression from nest mates, but lacking gemmae. A complementary way of assessing the importance of the gemmae is to study unmutilated individuals grown up in the presence of a gamergate. This can be achieved by coating the gemmae of unmutilated callows and placing them in their respective stock colony with a gamergate (type B females). The coating consisted of a layer of paint (the same paint used for marking the ants) applied on both gemmae immediately after eclosion. To control for the effect of transfer to another colony, we also transferred unmutilated workers without coating to their respective stock colonies (type C females). To control for the effect of the coating on the development of the bursa, we left some of the coated workers in the experimental colony (without gamergate) in which they had eclosed (type D females). When they reached the age of 15 days, all of these females were dissected and their bursa copulatrix was examined.

Dissections and histology

Individuals, taken from the treatments mentioned above, were killed by placing them in the freezer for a few minutes. Their abdomen was then dissected in Ringer–Jolly solution under a binocular microscope, to reveal the genital system.

After the bursa copulatrix was inspected, the bursa and the spermatheca were prepared for serial sectioning. They were fixed in 2% glutaraldehyde in a sodium-cacodylate buffer, postfixed in osmium tetroxide, dehydrated in a graded acetone-series, embedded in araldite and sectioned using a Reichert Ultracut E microtome. Semi-thin sections (1 µm) were stained with a methylene blue/thionine solution. Thin sections (70 nm) of the bursal epithelium were contrasted with uranyl acetate and lead citrate and viewed in a Zeiss EM 900 electron microscope. These sections were used to make a detailed description of the development of the structures with age. With the software SIGMA SCAN Pro 5, the following three measurements were taken for the spermatheca: the diameter of the spermathecal gland arms, the thickness of the reservoir epithelium close to the sperm duct (proximal or hilar epithelial thickness) and the thickness on the opposite side of the lumen (distal epithelial thickness). We took the two measures for the epithelial thickness because ant spermathecae have a differentiated region of columnar epithelium near the opening of the spermathecal duct (Wheeler and Krutzsch 1994; Gobin *et al.* 2001b). The mean values were obtained from measurements at five different places on the most representative section of each specimen.

We sectioned the bursa of eight individuals with gemmae and of five individuals without gemmae of different ages, and the spermatheca of 14 individuals with gemmae and 12 individuals without gemmae of different ages (Table 2).

For scanning electron microscopy, one spermatheca was dehydrated in formaldehyde dimethyl acetate and then critical point dried in a Balzers CPD 030 critical point drying device. It was coated with gold and observed in a Philips XL 30 ESEM scanning microscope.

We also inspected the bursa of the gamergate and one random worker from three colonies of *Diacamma pallidum* nesting in the forest soil around the Ulu Gombak Field Station, Malaysia (collected in August 2002) to check if our findings might apply to other species of the genus *Diacamma*. These six

individuals of unknown age were dissected 15 days after collection.

Results

Bursa copulatrix

The dissections revealed that the bursa copulatrix only develops into a functional structure in the unmutated workers. In these individuals, the bursa is discernible under a dissecting microscope from the age of 10 days and reaches its final size and coloration at the age of 15 days. It appears as a bi-lobed, transparent pouch in the posterior end of the abdomen, lined on the interior side with a dark brown cuticle. It is situated dorsally on the oviduct, with its lumen connecting to that of the oviduct (Fig. 1A). In the 32 mutilated workers, however, such a voluminous pouch with a dark interior was never observed. The same outcome was observed in the *D. pallidum* gamergates ($n = 3$) and workers ($n = 3$): only the gamergates had a developed bursa.

To study the development of the bursa copulatrix in more detail, we examined serial sections through the bursa of individuals with and without gemmae of different ages (Table 2). Sections of 1-day-old individuals, mutilated or unmutated, similarly show the precursor of the bursa as a folded epithelium (thickness about 80 µm), continuous with the dorsal wall of the oviduct (Fig. 1B). The initial cuticle that lines the lumen of this pouch is visible as a very thin black line. With increasing age, secretions from the bursal epithelium of unmutated individuals build up a very thick cuticular cover on the luminal side. Transmission electron micrographs show numerous microvillar extensions of the epithelial cells protruding into the cuticular matrix, indicating this secretory activity (Fig. 1D). Between 10 and 15 days after eclosion of the worker, the cuticular layer reaches its final and maximum thickness (varying between 80 and 300 µm, depending on the part of the bursa considered) (Fig. 1A). The whole organ is then about 800 µm wide and 700 µm high. In workers that lose their gemmae, however, the epithelium degenerates between the 5th and the 10th day after eclosion. After 10 days no cells are visible anymore, except for an occasional nucleus enclosed between the basal membrane and the cuticle. The degradation of the bursa thus seems irreversible. The remnant of the bursa is visible on sections as a collapsed cuticular expansion of the oviduct (Fig. 1C). Dissections of 15 mutilated workers from colonies in which the gamergate was absent for at least 2 weeks showed that this degeneration is indeed irreversible: these workers laid eggs but none of them developed the bursa copulatrix.

Experimental manipulation and development of the bursa copulatrix

Of the 10 artificially mutilated females (type A), 6 developed a bursa, and 4 did not. This indicates that the removal of the

Table 2 Number of naturally mutilated and unmutated *Diacamma* sp. workers dissected to study structure and development of bursa copulatrix and spermatheca

Age (days)	Bursa copulatrix		Spermatheca	
	Workers with gemmae	Workers without gemmae	Workers with gemmae	Workers without gemmae
1	3 (1)	6 (1)	(1)	(4)
5	3 (1)	8 (1)	(2)	(4)
10	3 (2)	6 (1)	(2)	(2)
15	5 (2)	6 (1)	(2)	(1)
30	13 (2)	6 (1)	(7)	(1)

Numbers in parentheses indicate how many of these individuals were used for histological sectioning.

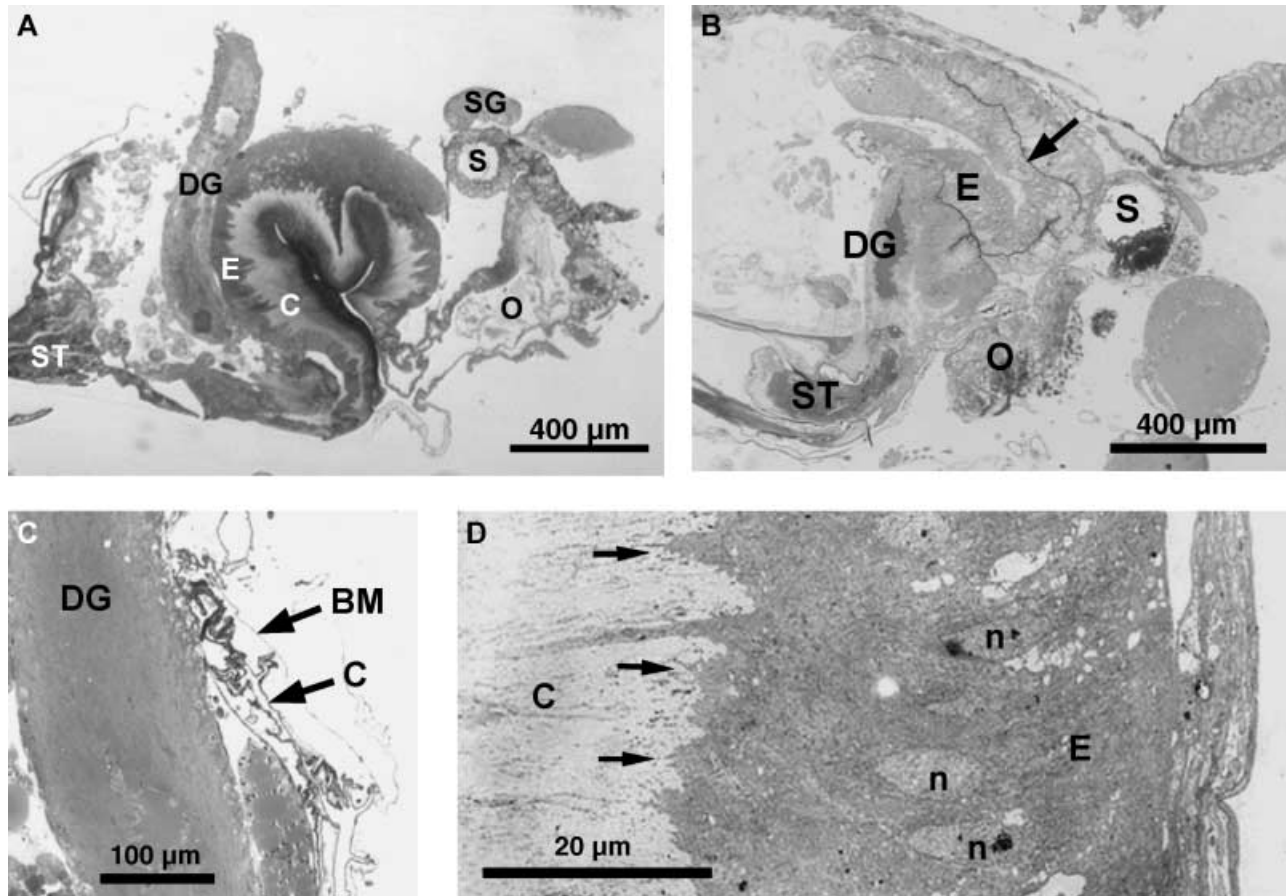


Fig. 1—**A.** Section through abdomen tip of 1-day-old unmutated *Diacamma* sp. worker (anterior side to the right), showing the precursor of the bursa as a folded epithelium (thickness about 80 μm). Arrow points at the bursal cuticle. (DG: Dufour gland; E: bursal epithelium; S: spermatheca; SG: spermathecal gland; ST: sting base; O: oviduct). —**B.** Section through abdomen tip of 15-day-old unmutated female (anterior side to the right), showing the fully developed bursa copulatrix. The epithelium, continuous with the dorsal wall of the oviduct, secreted a very thick cuticular layer (C) (thickness varying between 80 and 300 μm , depending on the location). (C: bursal cuticle; DG: Dufour gland; E: bursal epithelium; S: spermatheca; SG: spermathecal gland; ST: sting base). —**C.** Section through the abdomen tip of 15-day-old mutilated female (anterior side to the right), showing the remnant of the bursa: a collapsed cuticular expansion of the oviduct. (BM: basal membrane of the bursal epithelium; C: bursal cuticle; DG: Dufour gland). —**D.** Transmission electron micrograph of the boundary between epithelium and cuticle in the bursa copulatrix of a 30-day-old worker with gemmae. Arrows point to the numerous microvillar extensions of the epithelial cells. (C: cuticle; E: epithelium; n: nuclei of epithelial cells).

gemmae alone may impede the development of this organ, but this effect is not complete and it needs to be associated with aggression and/or gamergate presence.

Of the 12 coated females transferred to their respective stock colony (type B), 10 were killed and dismembered by their nest mates. Two callows survived the experiment with their gemmae still attached to their body, but they did not have a developed bursa, also suggesting that the gemmae are not the sole factor regulating bursa development. The intense aggression towards these callows can hardly be explained by a change in colony odour, as 16 of the 17 type C females did survive the transfer to their respective stock colony. These uncoated females were all successfully mutilated by their nest mates and did not develop a bursa. We suppose that the type

B callows succumbed to the persistent aggression of nest mates trying to remove the coated gemmae. The coating itself had no effect on the development of the bursa: 18 of the 19 type D females (coated gemmae, reared in the absence of a gamergate) had a fully developed bursa, one female died.

Spermatheca

In unmutated and virgin females of *Diacamma* sp., the spermathecal reservoir is spherical with an average diameter of 220 μm (Fig. 2A). The wall consists of a cylindrical epithelium (thickness about 30 μm) with a thin cuticle on the luminal side (thickness approximately 0.5 μm) (Fig. 2B). We did not find evidence of a thickened spermathecal epithelium

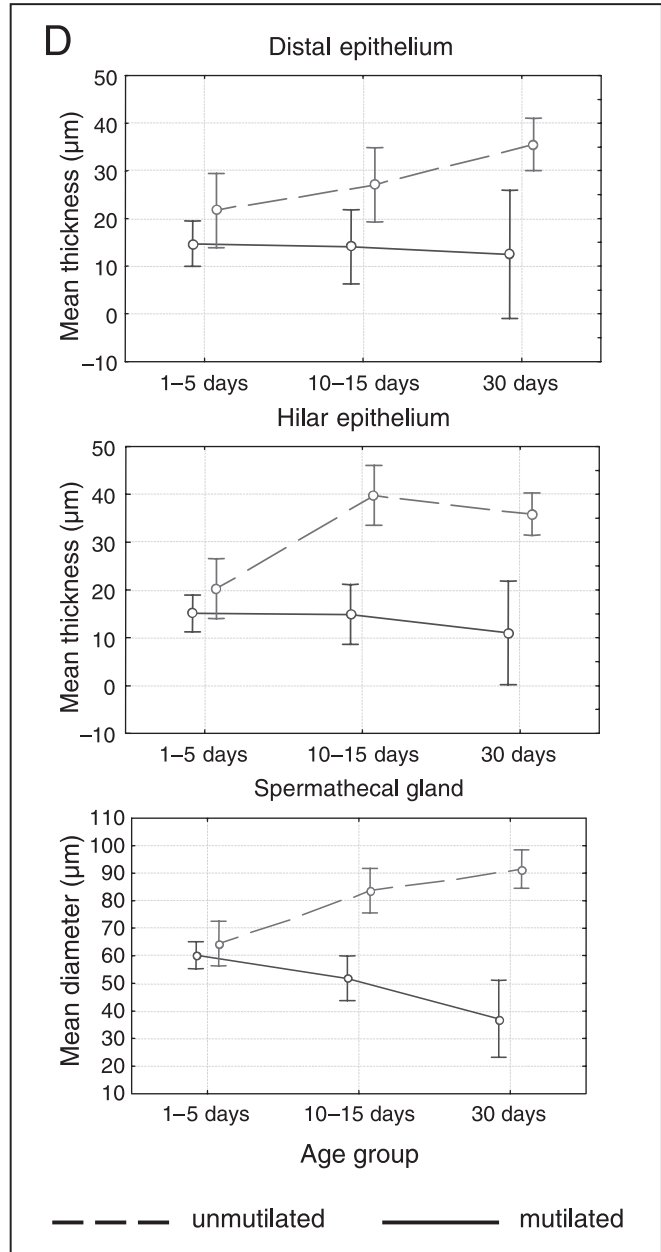
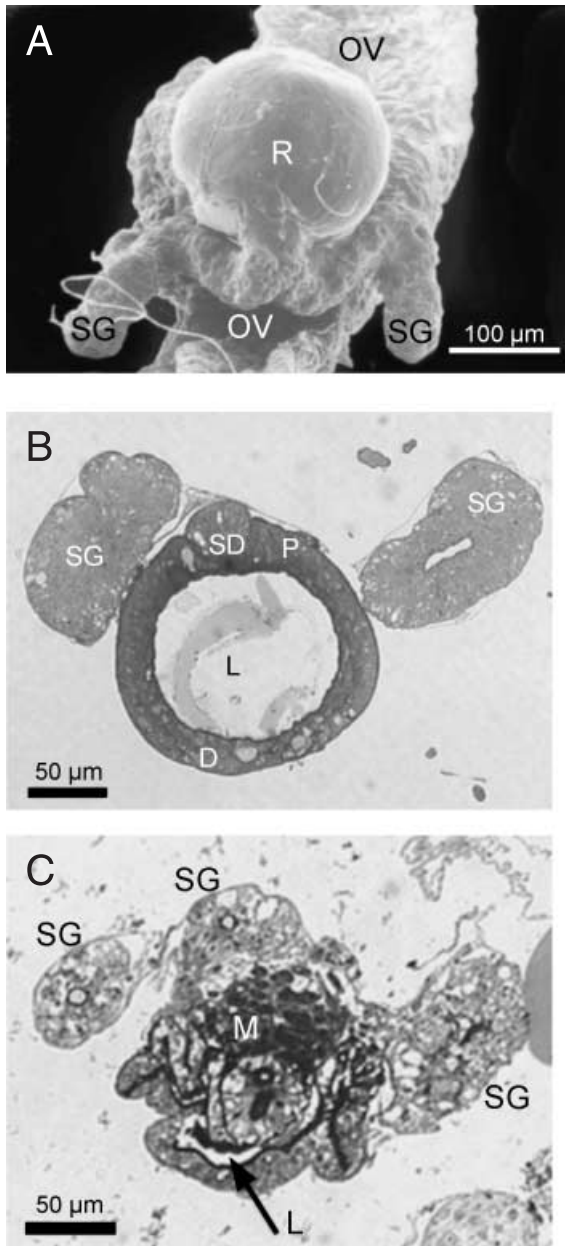


Fig. 2—**A**. Scanning electron micrograph of the spermatheca of a 30-day-old unmutilated *Diacamma* sp. worker (viewed from anterior side). The spermathecal gland (SG) consists of two coiled arms. (SG: spermathecal gland; OV: oviduct; R: spermathecal reservoir). —**B**. Cross-section through the spermatheca of a 30-day-old unmutilated worker. The spermathecal reservoir is ball-shaped with an average diameter of 220 µm. The wall consists of a cylindrical epithelium (average thickness 30 µm) with a thin cuticle on the luminal side. (D: distal side of the reservoir; L: lumen of the spermatheca; P: proximal or hilar side of the reservoir; SD: spermathecal duct; SG: spermathecal gland). —**C**. Cross-section through the spermatheca of a 30-day-old mutilated worker. The reservoir collapsed during the embedding process. (L: lumen of the spermatheca; M: muscles surrounding the spermathecal duct, representing the sperm pump; SG: spermathecal gland). —**D**. Graphs showing the evolution of three parameters of the spermatheca in function of age, for unmutilated (dashed line) and mutilated (full line) individuals. Values are means ± 1SD. For sample sizes: see Table 2.

near the entrance of the spermathecal duct. The accessory gland of the spermatheca is situated dorsally with regard to the reservoir. The gland consists of two arms whose central ducts come together in a common gland duct just before

joining the spermathecal duct (Fig. 2A). Both arms are tortuous so that multiple sections through the gland may show up on a transverse section (Fig. 2B,C). The external diameter of the glandular arms ranges from 70 to 90 µm. The lumen

has a diameter of approximately 10 μm and shows a transparent content. The glandular tissue consists of many secretory units, belonging to the highly derived Class 3 epidermal glands described in Noirot and Quennedey (1974). Each secretory unit consists of two cells: a secretory cell, which produces the secretion, and a duct cell that transports the secretion from the secretory cell to the gland lumen. Transverse sections through a glandular arm show about 10 of these units. Situated near the gland lumen is a second ring of smaller nuclei (average diameter 5 μm) with very granular nucleoplasm, belonging to epithelial cells lining the lumen.

In workers without gemmae, from the age of 10 days the spermathecal reservoir collapses during the tissue preparation (Fig. 2C), indicating that the structure loses its rigidity. The outline of the spermathecal gland arms is very irregular as opposed to workers with gemmae where it is nicely rounded (Fig. 2B,C). The content of the gland duct also differs between the two types of females: it stains clear in individuals with gemmae, while the lumen of the gland in mutilated individuals stains much darker.

The measurements of the thickness of the reservoir epithelium and the diameter of the gland ducts were grouped into three age categories (1–5 days, 10–15 days and 30 days) to obtain a sufficient number of replicates per category. Figure 2D shows that the epithelium and the gland tend to grow thicker with age in unmutated individuals, and to decrease in thickness with age in mutilated individuals. The difference between the two types of females was initially not significant (1–5 days old) (t -test: $P > 0.05$), but it became larger and statistically significant for the hilar epithelial thickness and gland diameter 10–15 days after eclosion (t -test: $P < 0.05$), and also for the distal epithelial thickness 30 days after eclosion (t -test: $P < 0.05$).

Discussion

In the queenless ant *Diacamma*, workers are aggressively mutilated by the gamergate soon after eclosion, and mutilated workers never mate. New gamergates can only originate in colonies lacking a gamergate, where the first eclosing callow remains to be mutilated (Fukumoto *et al.* 1989; Peeters and Higashi 1989). Our dissections and histological sections of *Diacamma* sp. from Japan revealed that the female genital tract develops differently in mutilated and unmutated workers. The bursa copulatrix of unmutated workers develops into a voluminous solid structure through the secretion of a thick cuticular layer. This bursa will allow the establishment of a strong connection with the specialized and sharp male genitals during copulation, without injury to the female (Allard *et al.* 2002). The cuticular layer reaches its final and maximum thickness after 10–15 days, the age at which unmutated females start mating (Fukumoto *et al.* 1989). The bursal epithelium of mutilated individuals, on the other hand, degenerates in a few days without production of such a cuticle, leaving the worker permanently incapable of mating.

Similar changes were observed in *Diacamma pallidum* from Malaysia. For the spermatheca, the spermathecal wall and spermathecal gland of unmutated individuals grow significantly thicker than those of mutilated individuals within a few weeks after eclosion. Moreover, the gland ducts show a different content in the two types of females. The spermathecal reservoir and associated gland are essential for the conservation and activation of sperm cells (Wheeler and Krutzsch 1994). In the fictitious case that a mutilated female would receive sperm from a male, we hypothesize that she would be less competent than unmutated females for long-term sperm storage and sperm activation. To summarize, when unmutated females (future gamergates) normally start mating, mutilated females are morphologically incapable of copulating and less fit for storing and activating sperm. This is the first description of such a morphological differentiation between subordinate and dominant workers in a queenless ant. Future research must reveal if this phenomenon occurs in all *Diacamma* species and possibly in other queenless ants.

The regulation of sexual activity through mutilation of the gemmae in *Diacamma* is unique among ants and cannot easily be extrapolated to queenless ants in general. However, our results (especially those with type A females) suggest that the presence or absence of gemmae alone is not the sole factor determining the development of female genital tracts. Therefore, the regulatory system in *Diacamma* might be closely related to those in other queenless ants. Future studies must reveal how this particular system in *Diacamma* has evolved and how it is related to the regulatory systems in other queenless genera. It is almost impossible to understand the evolution of the unique system in *Diacamma* without knowledge of the proximate mechanisms.

Acknowledgements

We are grateful to Koen Collart for his help in making the histological sections. D. Allard and B. Gobin were funded by the Fund for Scientific Research Flanders and by OT-project 01.24 of the K.U. Leuven Research Fund. F. Ito was supported by Grants in Aid for Scientific Research from the JSPS numbers 11691130 and 14405036. K. Tsuji's work was funded by a Grant in Aid from the Japan Ministry of Education, Science and Culture number 13640626. We thank Rosli Bin Hashim and Batera from the Ulu Gombak field station, Malaysia, for their help during our stay.

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