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Original article

Vestigial spermatheca morphology in honeybee workers, *Apis cerana* and *Apis mellifera*, from Japan

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Abstract – Reduction of reproductive organs in workers is one of the most important traits for caste specialization in social insects. In this study, we investigated the morphology of the sperm storage organ, the spermatheca, in workers of the honeybees *Apis cerana* and *Apis mellifera* from Japan. All examined workers of the two species retain a small spermatheca. Ultrastructural observations indicate that the spermatheca of honeybee workers consists of conspicuous muscular layers which correspond with the sperm pump and that the epithelium surrounded by these muscular layers represents the spermatheca duct. As this epithelium shows intact cellular morphology, we conclude that the spermatheca duct and the sperm pump do not degenerate, whereas the spermatheca reservoir has completely degenerated. All workers of *A. cerana* and most workers of *A. mellifera* lack the spermatheca gland completely. A few workers of *A. mellifera* possess the opening of the spermatheca gland, but its structure seems to be degenerated. The spermathecal width including the muscular layers showed a weak, but significant positive correlation with ovariole numbers in *A. cerana*, but not in *A. mellifera*. The degree of spermatheca gland degeneration seems to be unrelated to ovariole number. The morphological features of degeneration in honeybee workers are different from those of the vestigial spermatheca in ant workers, indicating that morphological worker virginity has evolved in a different way.

spermatheca morphology / honeybee worker / caste dimorphism / reproductive constraint / degree of degeneration

1. INTRODUCTION

Eusociality has evolved many times among insects, and the degree of sociality shows a variety from primitive to high. In highly social species, the reproductive capacity of helpers is not only regulated by behavioral and physiological constraints as observed in primitively eusocial insects, but also by morphological and functional constraints of the reproductive organs, such as the ovaries and spermatheca. Caste differences of ovariole number and ovarian activity have already been well reported in many species (ants: Hölldobler and Wilson 1990; Khila and Abouheif 2008, 2010; wasps: Spradbery 1973; Kugler et al. 1976; bees: Snodgrass 1956; Tanaka and Hartfelder 2004). On the other hand, differences of spermatheca morphology between reproductives and nonreproductives have received more attention recently (Schoeters and Billen 2000; Gobin et al. 2006, 2008; Gotoh et al. 2008), although the degree of the caste differences imply significant meanings for the evolution of caste dimorphism. In order to produce female offspring, female individuals in social Hymenoptera need to mate and

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store the spermatozoa in their spermatheca. Without the spermatheca, however, females cannot participate in female production. The presence or absence of a functional spermatheca in nonreproductive females, therefore, is a very important trait for caste dimorphism.

In Hymenoptera, the spermatheca comprises a spermatheca reservoir, a spermathecal duct, and a pair of spermathecal glands. The spermatheca reservoir can be lined with a flattened epithelium or may be partly or entirely formed by a cylindrical epithelium. A muscular structure that acts as a sperm pump is located near the entrance of the spermathecal duct into the spermatheca reservoir. In some stenogastrine, polistine, and vespine wasp species which belong to Vespoidea, morphological degeneration of the spermatheca in nonreproductives have never been found, although nonreproductive females of polistine and vespine wasps never mate as far as we know (Gotoh et al. 2008). In some bumblebee species, the workers also retain an intact spermatheca morphology, but they lack PAS-positive epithelial cells, which indicates the presence of glycogen and other polysaccharides in the spermathecal duct in contrast to queens (Schoeters and Billen 2000). Among social Hymenoptera, spermatheca degeneration has been reported only in workers of honeybees and ants. In most ant species, workers lack the spermatheca completely. On the other hand, workers of part of the ponerine, ectatommine, and amblyoponine species retain a vestigial spermatheca, in which workers not only have a reduced spermatheca size, but also display a degenerate spermatheca morphology (Ito and Ohkawara 1994; Gobin et al. 2006, 2008). In ant queens, only the hilar region of the spermatheca reservoir develops columnar cells, whereas in ant workers with a vestigial spermatheca, such columnar epithelium is lacking. The presence of a columnar epithelium is, therefore, considered to be an important condition for sperm storage (Wheeler and Krutzsch 1994; Gobin et al. 2006, 2008). In honeybees, only some simple descriptions and an old histological study about the worker spermatheca in *Apis mellifera* have been reported (Adam 1912; Snodgrass 1956; Winston 1987). There is no clear histological study in the worker of *Apis* species, however, while the degree of spermatheca degeneration compared to the spermatheca morphology of honeybee queens is still unknown. Here, we examined the morphology of the spermatheca in workers of the Japanese honeybees *Apis cerana* and the European honeybees *A. mellifera* from Japan and compare it with a previous study of the spermatheca morphology in the queen of *A. mellifera* (Dallai 1975).

2. MATERIALS AND METHODS

2.1. Honeybees and sample preparation

In Japan, both the Japanese honeybee *A. cerana japonica* Radoszkowski and the European honeybee *A. mellifera* Linnaeus are found (Matsuura 1995). We collected a total of 237 foraging workers of both species between 2008 and 2012 in Aichi prefecture and in 2007 in Kagawa prefecture, Japan. The two species were identified by their hindwing veins (*A. cerana* has an extra vein; Alexander 1991). After the bees were anesthetized on ice, they were dissected in 1× phosphate-buffered saline (pH7.4) for scanning electron microscopy (SEM) and histological observations and for measurement of the spermatheca size. All samples were checked for the presence or absence of the spermatheca.

2.2. SEM observations

Six samples (five *A. cerana* and one *A. mellifera*) fixed in FAA (ethanol/formalin/acetic acid, 16:6:1) were dehydrated in a graded ethanol series and were critical point dried using CO_2 and coated with gold by ion sputtering (EIKO IB-3) for observation in a Hitachi S800 scanning microscope.

2.3. Histological and ultrastructural observations

For brief histological observations, we fixed 52 honeybee abdomens (41 *A. cerana* and 11 *A. mellifera*) in FAA and dehydrated these in a graded ethanol series before embedding in paraffin. Longitudinal serial sections were made at a thickness of 4 μm and stained with hematoxylin and eosin. One hundred thirty-three samples (72 *A. cerana* and 61 *A. mellifera*) were fixed in 2 % glutaraldehyde and transferred to 0.05 M Na–cacodylate buffer with 150 mM saccharose. After postfixation in 2 % osmium tetroxide, tissues were dehydrated in a graded acetone series, embedded in araldite, and cut into 1-μm serial longitudinal sections. Semithin sections stained with methylene blue were observed with a Leica DMRB microscope and photographed with a 3CCD digital camera (Victor KY-F75). Thin sections (70 nm) were made with a Leica EM UC6 ultramicrotome and double-stained with uranyl acetate and lead citrate (Gotoh et al. 2008). Sections were observed with a Zeiss EM 900 electron microscope.

2.4. Spermatheca size measurement by differential interference contrast microscopy

To measure the spermatheca width and observe the general spermatheca morphology, we used differential interference contrast microscopy for 24 *A. cerana* and 22 *A. mellifera* workers. This method is useful to find vestigial spermatheca gland-like structures easily. The spermatheca together with the common oviduct was dissected and put into a droplet of 1× phosphate-buffered saline (pH 7.4) and covered with a coverslip to observe the spermatheca morphology from above. The specimens were observed with a differential interference contrast microscopy (Leica DMRB) and photographed with a 3CCD digital camera (Victor KY-F75). Spermatheca width was measured using the Image J software (http://rsbweb.nih.gov/ij/).

3. RESULTS

The spermatheca is located posteriorly to the last abdominal ganglion. All worker honeybees examined in this study possess a spermatheca (142 *A. cerana* and 95 *A. mellifera*). The morphology of the spermatheca is very similar in workers of both species. The spermatheca is covered by a few tracheas (Figure 1a–d). The distal tip of the spermatheca is swollen by the development of muscular tissue surrounding the spermatheca epithelium region (Figure 1a–c). Without the muscular layers, the spermatheca epithelium region has a Y shape when viewed from above (Figure 3a–c). From a lateral view, the spermatheca is shaped like a flattened sac (Figure 1b). The entire spermathecal epithelium is formed by a single layer of columnar cells with a variable thickness from about 10 to 30 μm in both species (Figure 1b–d). The epithelium is lined by a cuticle with a thickness between 2 and 8.5 μm. The columnar cells at the proximal part of the spermatheca contain abundant mitochondria and well-developed microvilli (Figure 2a–c). At the distal part of the spermatheca, myofilaments running perpendicular to the cuticle transmit their pulling forces onto microtubules that occur in the epithelial cells (Figure 2d). The myofilaments and microtubules are connected via horseshoe-shaped hemidesmosomes (Figure 2e); the microtubules apically attach to the cuticle, where hemidesmosomes can also be seen. In between the muscle fibers, nerve fibers could be seen occasionally.

Observation by differential interference contrast microscopy revealed that all 24 workers of *A. cerana* and 17 of 22 workers of *A. mellifera* lack the spermatheca gland completely, without any trace of the spermatheca gland opening (Figure 3a). On the other hand, we found an extension of the lumen-lining cuticle that penetrates the epithelium and muscle layers in five of 22 workers of *A. mellifera* and considered this structure to represent the opening of the spermathecal gland (Figure 3b, c). This cuticular extension ends within the surrounding muscle layers in four of five workers (Figure 3b). In only one worker of *A. mellifera*, this cuticular structure elongates beyond the muscle layers, with its distal part surrounded by cells with a round shape (Figure 3c). Histologically, the cells surrounding the muscle layers contain several nuclei and their shape is distorted (Figure 3d). The mean number of ovarioles of *A. mellifera* workers is 8.74±6.54 (range, 2–36; median, 7; n=35). One worker had 36 ovarioles, which is the largest ovariole number among our samples. This individual displayed an elongated cuticle layer extending beyond

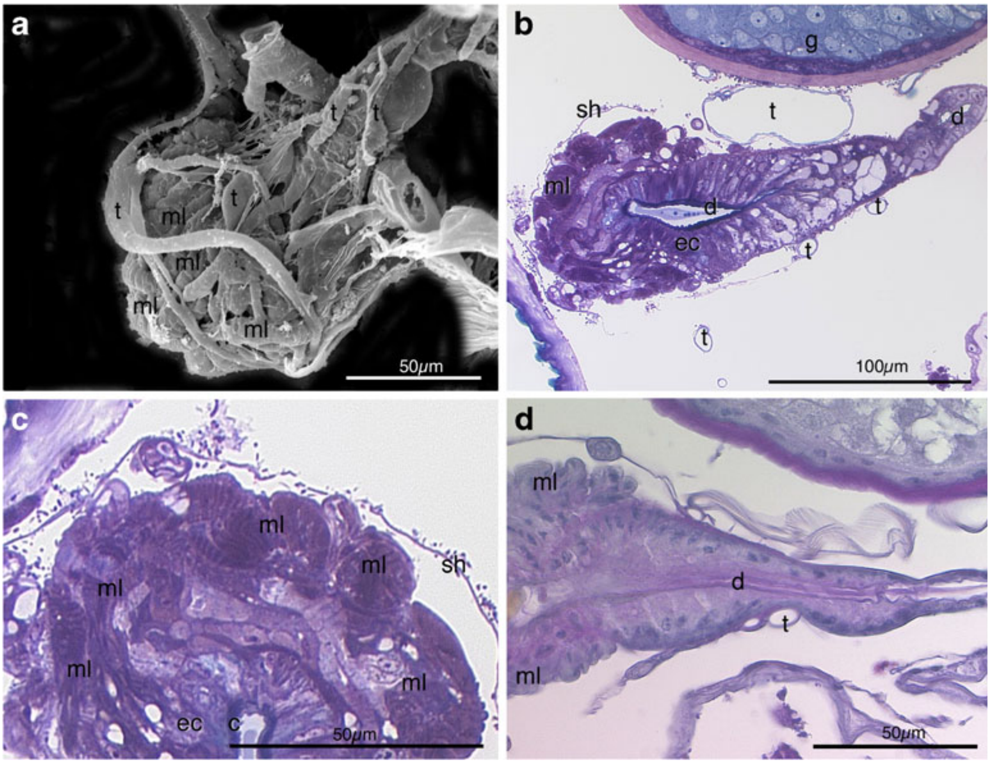
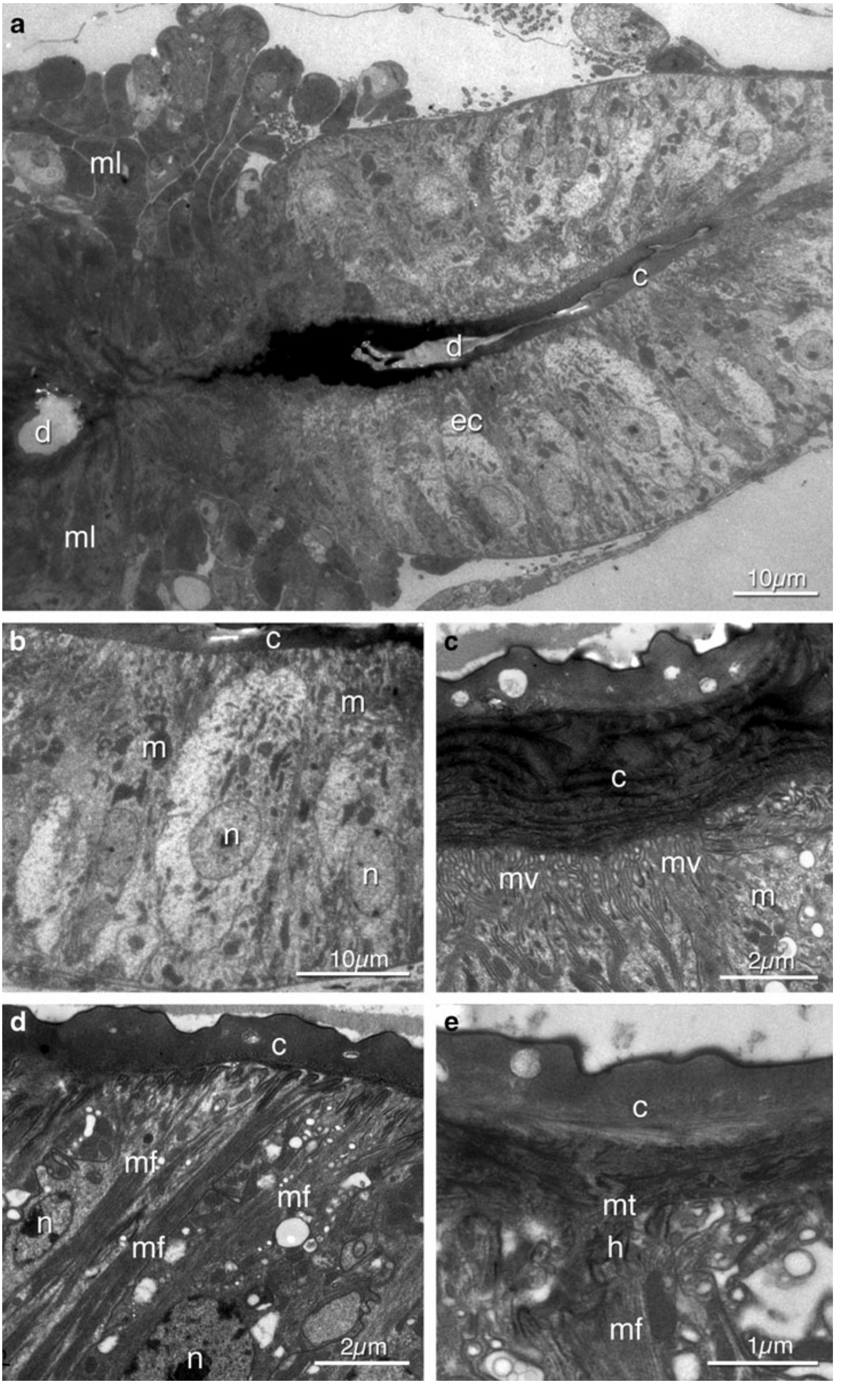


Figure 1. Spermatheca morphology in *A. cerana* workers. Pictures of the spermatheca from dorsal view by scanning electron microscopy (**a**). Longitudinal sections of the spermatheca (**b**) and details of the distal (**c**) and proximal region (**d**) of the spermatheca. *c* cuticle, *d* duct, *ec* epithelial cells, *g* ganglion, *ml* muscle layers, *sh* sheath, *t* trachea.

the muscular layers, and besides having the highest ovariole number, thus also showed the most recognizable trace of a spermatheca gland, but the other workers with the opening of the spermatheca gland possess a moderate number of ovarioles (ovariole numbers of these four workers are 4, 8, 13, and 15; Figure 4b). Both *A. mellifera* workers with and without the opening of the spermatheca gland were obtained from the same small collection site. In *A. cerana* examined here, the mean number of ovarioles is 12.13±4.06 (range, 5–36; median, 11; n=92), and all *A. cerana* workers, even the worker with 36 ovarioles, do not have the spermatheca gland-like structure. The spermatheca width including the muscle layers is 162.92± 32.16 μm (mean±SD) in *A. cerana* (n=21) and 174.17±62.26 μm (mean±SD) in *A. mellifera* (n=21). The spermathecal width with the muscular layers and the number of ovarioles show a weak correlation in *A. cerana* (Pearson's correlation coefficient, n=21, r=0.44, P<0.05; Figure 4a), but no significant correlation is found in *A. mellifera*

Figure 2. Ultrastructure of the spermatheca in honey-▶ bee workers. **a** Overview of the spermatheca. Details of the proximal part (**b**, **c**) and apical region (**d**, **e**) of the spermatheca. **c** and **e** are detailed images of **b** and **d**, respectively. **a**, **b** *A. cerana* and **c**–**e** *A. mellifera*. *c* cuticle, *d* duct, *ec* epithelial cells, *h* hemidesmosome, *m* mitochondria, *ml* muscle layers, *mf* myofilaments, *mt* microtubules, *mv* microvilli, *n* nuclei.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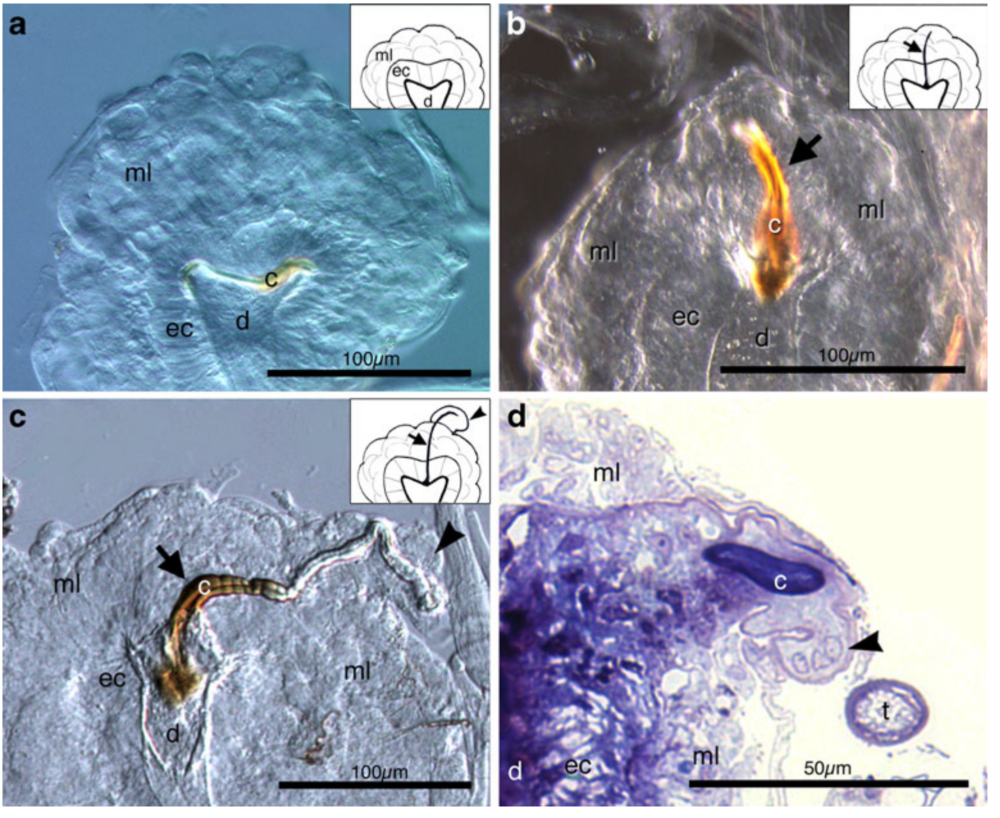

Figure 3. Variation of degenerated spermatheca gland morphology in honeybee workers observed by differential interference contrast microscopy (**a**–**c**) and longitudinal sections (**d**). Schematic indications of spermatheca morphology are shown in the upper right corner (**a**–**c**). All *A. cerana* (**a**) and most *A. mellifera* workers examined here lack the spermatheca gland completely. In *A. mellifera*, some workers retain only the opening of the spermatheca gland (**b**) and very exceptionally display the cuticular extension elongated beyond the surrounding muscular layer (**c**, **d**). *Arrows* indicate the opening of the spermatheca gland and *arrowheads* point to the spermatheca gland-like structure. *c* cuticle, *d* duct, *ec* epithelial cells, *ml* muscle layers, *t* trachea.

(Pearson's correlation coefficient, n=21, r= 0.20, P=0.379; Figure 4b).

4. DISCUSSION

This is the first detailed histological study of the spermatheca in honeybee workers. We confirmed that all 237 examined worker honeybees do possess a spermatheca. The general spermatheca morphology of honeybee workers is slightly unusual among hymenopteran species. The external morphology by SEM observation shows that the distal tip of the spermatheca shows a spherical shape, which is formed by thick muscular layers surrounding the epithelium region. The spermatheca without the surrounding muscular layers is shaped like a flattened sac. This feature is different from the spermatheca reservoir in other Hymenoptera and honeybee queens, in which a swollen-shaped spermatheca reservoir is found (Dallai 1975; Wheeler and Krutzsch 1994; Schoeters and Billen 2000; Martins et al. 2005; Gotoh et al. 2008). The spermatheca is formed by

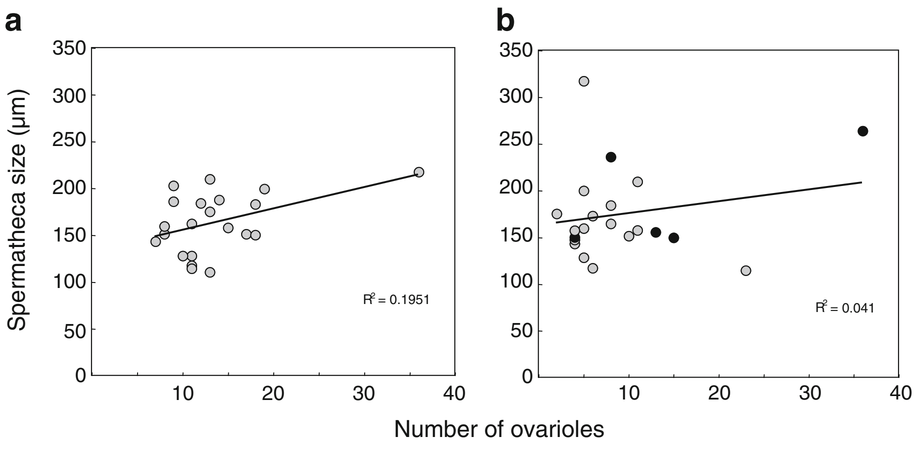

Figure 4. Spermatheca size with muscular layers in relation to number of ovarioles in *A. cerana* (**a**) and *A. mellifera* (**b**). *Filled circles* indicate the value of workers with the opening of the spermatheca duct in *A. mellifera*.

columnar epithelial cells, and abundant myofilaments in the muscle fibers connect through hemidesmosomes to parallel bundles of microtubules that occur inside the epithelial cells at the distal part of the spermatheca. This configuration allows the muscle fibers to transmit their pulling forces onto the cuticle, as is typical for arthropods in general (Caveney 1969; Lai-Fook 1967; Billen 1982, 2006). These features strongly indicate that this structure is equivalent to the sperm pump described in the queen spermatheca of *A. mellifera* (Dallai 1975), and abundant mitochondria and microvilli are observed in the columnar epithelium at the proximal part of the spermatheca which are similar in morphology to the epithelium of the spermatheca duct and the spermatheca reservoir in *A. mellifera* queens (Dallai 1975; Martins and Serrão 2002). Since queen honeybees lack muscles surrounding the spermatheca reservoir, we conclude that the spermatheca reservoir is completely degenerated and that the remaining epithelial tissue corresponds with the spermatheca duct. The cells of this epithelium maintain an intact morphology in all honeybee workers examined. Furthermore, Dallai (1975) described that the diameter of the spermatheca duct including the sperm pump in queens of *A. mellifera* is 170 µm. This is close to that of the workers examined here (about 163 µm in *A. cerana* and about 174 µm in *A. mellifera*). These observations suggest that the morphology and the size of the spermatheca duct and the sperm pump are not reduced in honeybee workers examined here. All *A. cerana* and most *A. mellifera* workers lack the spermathecal gland completely. Five of 22 workers of *A. mellifera* retain the opening of the spermatheca gland. The spermatheca gland-like structure in the workers, however, looks much shorter than the spermatheca gland in *A. mellifera* queens (Figure 1 in Dallai 1975). Furthermore, the spermatheca gland-like structure outside of the muscle layers does not seem to be a functionally active tissue, as it has a distorted shape and also lacks the usual composition of the spermatheca gland with secretory cells and duct cells, as is characteristic for the majority of Hymenoptera (Pabalan et al. 1996). We, therefore, conclude that, in honeybee workers, the spermatheca reservoir and the spermathecal gland in the portion distal to the muscular layers of the sperm pump are degraded (Figure 5). We also found that a few tracheas occur around the spermatheca, which is in contrast to the extensive tracheal network surrounding the spermatheca reservoir in *A. mellifera* queens (Poole 1970; Dallai 1975). This

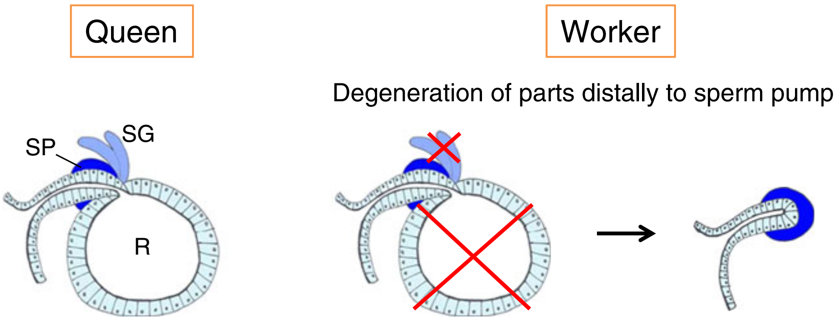

Figure 5. Schematic illustration of spermatheca morphology in honeybee queens and workers. *R* reservoir, *SG* spermatheca gland, *SP* sperm pump.

suggests that the tracheal system degenerates with regression of the spermatheca reservoir in honeybee workers.

In our study, some workers of *A. mellifera* maintain the opening of the spermatheca gland, but the majority lacks the spermatheca gland completely. In some old literature, the presence of the spermatheca glands in workers of *A. mellifera* has been reported (Adam 1912; Hambleton 1928; see Kapil 1962). Kapil (1962), however, reported that workers of *Apis indica* (the oriental honeybee) lack the spermathecal gland. The presence and size of the spermatheca in *A. mellifera* subspecies has been described well, especially in workers of the Cape honeybee *A. mellifera capensis* which are social parasites and can produce diploid eggs by thelytokous parthenogenesis. Jordan et al. (2008) described that only 8.3 % workers of *A. mellifera capensis* retain the spermatheca, while workers of *A. mellifera scutellata* lost the spermatheca completely. Phiancharoen et al. (2010) reported that the percentage of Cape worker honeybees with a spermatheca shows geographical differences from 0.4 to 19.2 %. Spermathecal size also shows variation. Hepburn and Radloff (2002) summarized the literature reporting on spermathecal size in Cape honeybee workers. Worker bees have a spermatheca with a diameter of about 500 μm from Cape peninsula, between 65 and 650 μm in bees from Stellenbosch, and between 150 and 590 μm in bees from the southern Cape region. This variation in spermathecal size is considered to depend on the bees' nutritional condition (Beekman et al. 2000; Calis et al. 2002; Allsopp et al. 2003). From our study and previous studies, we suggest that morphology and size of the spermatheca is variable in European and Cape honeybee workers, depending on geographical and nutritional factors. In this study, we collected both *A. mellifera* workers with and without the opening of the spermatheca gland from the same area, suggesting that nutrition during the immature stages or genetic background may affect the degree of spermatheca degeneration.

In a previous study, a positive correlation between spermatheca size and ovariole number was found in *A. mellifera capensis*, *A. mellifera scutellata*, and their hybrids (Jordan et al. 2008). In this study, spermathecal size with the muscle layers and the number of ovarioles shows a weak correlation in *A. cerana* but not in *A. mellifera*. In *A. mellifera*, workers with the most developed spermatheca gland-like structure have the largest number of ovarioles. The other workers with the opening of the spermatheca gland, however, have a moderate number of ovarioles. This suggests that the degree of degeneration in ovariole number and the spermatheca are not correlated in *A. mellifera*.

From our study of the spermatheca morphology, we need to alert honeybee researchers when they measure the spermatheca size. When honeybee workers loose the spermatheca reservoir completely, one may measure the width including the sperm pump and the spermatheca duct as a spermatheca width. However, if honeybee workers still maintain the spermatheca reservoir in a different region, researchers may measure spermatheca reservoir width without muscular layers as spermatheca width. Measurements of the spermatheca reservoir and the spermatheca duct with sperm pump cannot be compared to each other because they are different parts of the spermatheca, so that it is necessary to understand spermatheca morphology before measurement.

To date, caste dimorphism in the spermatheca is found only in ants and honeybees among social Hymenoptera (Snodgrass 1956; Schoeters and Billen 2000; Gobin et al. 2006, 2008; Gotoh et al. 2008). Workers of most ant species lack the spermatheca. On the other hand, ant workers with a vestigial spermatheca in some species maintain the muscular layers of the sperm pump, the spermatheca gland, the spermatheca duct, and the spermatheca reservoir, but the hilar columnar epithelium with abundant mitochondria and microvilli in the spermatheca reservoir is degenerated and is converted into inactive squamous epithelial cells (Gobin et al. 2006, 2008). Honeybee workers examined here lack the spermatheca reservoir completely and have no or just a vestigial spermatheca gland. From a comparison of the spermatheca morphology between ants and honeybees, we conclude that the spermatheca reservoir seems to degenerate first. In ants, to date, there are no reports of degenerated spermatheca morphology lacking the spermatheca reservoir or the spermatheca gland like honeybee workers. On the other hand, we never found honeybee workers with vestigial spermatheca reservoir consisting of morphologically inactive epithelium as the spermatheca of the worker in some ant species (Gobin et al. 2008). Interestingly, morphological variation of the worker spermatheca within species has never been reported in ants (Gobin et al. 2006, 2008; Gotoh, personal observation; Ito, personal observation). In contrast, the degree of spermatheca degeneration varies among workers of *A. mellifera*, as shown in our observations and some studies of Cape honeybees (Jordan et al. 2008; Phiancharoen et al. 2010). Workers of *A. cerana* and *A. mellifera* also show a great variation of ovariole number in this study. In ants, only a few species show a large variation of ovariole numbers among workers; however, the range of variation is apparently smaller than that in honeybee workers (Hölldobler and Wilson 1990; Ito et al. 1994; Ito 2010). Different from ants, it is considered that the degree of degeneration in reproductive organs may be very labile in workers of honeybees. Morphological worker sterility is considered to be an adaptive trait for social insects to prevent intracolonial conflict and decrease of colony efficiency (Khila and Abouheif 2010). It is thus enough to lose the reproductive functions though it is not necessary to evolve regression of the worker reproductive organs in a similar way among taxa. Workers of ants and honeybees reduced their spermatheca morphology in their own way and these morphological degenerations such as absence of columnar cells of spermatheca reservoir in ants and loss of the reservoir and degeneration of the spermatheca gland in honeybees may be easy to evolve for each species.

Since early times, researchers have this question: “Why do prominent caste differences occur in higher social insects although all females have a similar genetic background?” Recent “socio evo-devo” studies are engaged to answer this question using molecular techniques. *Apis* species are good material to study this theme because they have been treated as a model organism among social insects and the whole genome of *A. mellifera* has been revealed (Honeybee Genome Sequencing Consortium 2006). For these advanced studies, the present study will be a useful source of information of caste differences in honeybees.

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Morphologie de la spermathèque atrophiée chez les ouvrières des abeilles, *Apis cerana* et *A. mellifera* au Japon.

Morphologie / spermathèque / ouvrière / dimorphisme de caste / contrainte reproductive / degré de dégénération

Morphologie der rudimentären Spermatheka bei Arbeiterinnen der Honigbienen *Apis cerana* and *A. mellifera* aus Japan

Spermathekamorphologie / Honigbienenarbeiterin / Kastendimorphismus / limitierender Faktor der Reproduktion / Grad der Gewebedegeneration

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