

Sperm transfer during mating in the pharaoh's ant, *Monomorium pharaonis*

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Abstract. Sperm transfer in the pharaoh's ant *Monomorium pharaonis* (L.) is studied by making longitudinal sections through the gasters of mating pairs fixed in copula. Sperm is transferred inside a spermatophore similar to those found in two other ants, *Diacamma* sp. from Japan and *Carebara vidua*. Sharp teeth-ridges are present on the penis valves and, during copulation, these teeth make contact with a thick and soft cuticular layer covering the bursa copulatrix. This ensures an attachment long enough for the successful transfer of the spermatophore to the right position inside the female oviduct. The thick cuticle also protects the queen from serious damage by the male's sharp claspers. After a first successful copulation, sperm is still present inside the male's seminal vesicles, suggesting that males can mate multiply. Additional experiments, where single, initially virgin males are presented to several virgin females, confirm this.

Key words. Formicidae, genital spines, mating, spermatophore.

Introduction

Ants are renowned for their species richness (Bolton, 1995) and immense diversity in life histories, including mating systems. Hölldobler & Bartz (1985) recognized two major patterns in mating behaviour, which they termed the 'female calling syndrome' and the 'male aggregation syndrome', based mainly on the physical distribution of males and females during the period of mating activity, and on female mating frequency. If more parameters are taken into account, such as male mating frequency, sex ratio, competition, partner choice, sperm number, genital structure, mode of sperm transfer, etc., the pattern becomes more complicated, but the selective forces shaping ant mating systems are more likely to be identified.

The mode of sperm transfer during mating is a little explored topic in ants. However, the transfer of gametes is the crucial element in any mating process. Robertson (1995)

studied sperm transfer in the myrmicine *Carebara vidua*. After a short dispersal flight, females of *C. vidua* attract foreign males with a sex pheromone (Robertson & Villet, 1989). Each female can mate with up to four males, in copulations that last approximately 4 min each (Robertson & Villet, 1989). Male and female are connected by the genitalia: the male aedeagus (intromittent organ) fits into the female bursa copulatrix, with the digitus and cuspis fitting around the female's last gastral tergite (Robertson, 1995). For the nomenclature of male genitalia, Michener (1956) is followed: digitus and cuspis form a pincer-like extension of each volsella; the volsellae are two appendages of the male external genitalia, surrounding the intromittent organ. The male *C. vidua* transfers his sperm to the female in a spermatophore, formed by a secretion with high mucus-content from the male accessory glands (Robertson, 1995). No information on sex ratio or male mating frequency in *C. vidua* is provided here but, because females mate multiply, they might be the limiting sex, providing the males with very limited mating opportunities. Allard *et al.* (2002) studied sperm transfer in the ponerine *Diacamma* sp. from Japan. Virgin reproductive workers of this queenless species (one per colony) leave the nest intermittently to attract males with a sex pheromone (Fukumoto *et al.*, 1989; Nakata *et al.*, 1998). Males from

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foreign colonies land near the calling female. The female accepts mounting by a single male, and drags him into the nest in copula (Fukumoto *et al.*, 1989). The pair remains connected by the genitalia for a period lasting from several hours to 2 days, during which the male is killed and dismembered by the female and her nestmates (Fukumoto *et al.*, 1989). Consequently, males mate only once. The females of *Diacamma* sp. also mate only once. The strong connection between sexual partners is realized by the male inserting his sharp penis valves inside the female bursa copulatrix, clasping the female's last sternite between digitus and cuspis, and inflating his penis inside the female genital chamber (Allard *et al.*, 2002). The male of *Diacamma* sp. also transfers his sperm in a spermatophore, a sheath of gelatinous material produced by its accessory glands (Allard *et al.*, 2002).

To gain an understanding of how natural and sexual selection shaped those mating systems, it is necessary to investigate mating behaviour and sperm transfer in more ant species with varied mating systems. In the present study, mating pairs of the myrmicine *Monomorium pharaonis* (L.), the pharaoh's ant, are used. Originally, *M. pharaonis* was an Old World tropical species but, during the last 100 years, it has also become established (as a pest) in permanently-heated buildings in temperate regions throughout the world (Edwards, 1986). Nuptial flights do not occur in this species. The polydomic colonies disseminate by budding (Edwards, 1987). Sex ratios are strongly female-biased: the number of queens produced is three- to four-fold greater than the number of males (Peacock, 1951; Petersen & Buschinger, 1971). Petersen & Buschinger (1971) studied the mating behaviour of *M. pharaonis* in detail. Mating activity occurs when a colony produces sexual imagoes. The intervals between the productions of sexuals may vary from approximately 3 months (Petersen-Braun, 1975) to 1 year (Edwards, 1987). Collective mating episodes take place throughout the day every 3–4 h. Each bout of mating activity is started by a few excited males, and lasts for 15–30 min. The winged but flightless queens are inseminated in the nest or on worker-trails in the vicinity of the nest. Young virgin queens produce a short-range sex pheromone in the Dufour gland and bursa copulatrix, which attracts males from a short distance (approximately 6 cm) and stimulates male mating behaviour at a distance of 1–2 cm (Hölldobler & Wüst, 1973). Copulations last 40–60 s, during which females that started mating outside quickly enter the nest, dragging the male after them. Copulation normally ends abruptly. In rare cases, copulations that last for more than 4 min are ended by the female bending sideways and biting the male. Females mate only once; males inseminate two to four females, and rarely one (Petersen & Buschinger, 1971).

Monomorium pharaonis contrasts with *C. vidua* and *Diacamma* sp. in several respects. It presents strongly female-biased sex ratios at the location of mating, multiple-mating males and relatively short copulations. Differences in the modalities of sperm transfer might be expected to be linked to the differences in these important

features of mating systems. Sperm transfer in the pharaoh's ant is studied using morphological techniques. Additionally, the present study investigates whether males of this particular population, coming from the same stock used by Petersen-Braun and Buschinger more than 30 years ago, still inseminate multiple females.

Materials and methods

Sexuals of *M. pharaonis* were reared and a method for inducing mating was developed. Mating pairs were embedded in Araldite resin (Polysciences, Inc., Warrington, PA) and sectioned longitudinally to examine the mode of sperm transfer and the function of the genitalia during the mating process.

Rearing of sexuals

Sexual pupae of *M. pharaonis* were collected from stock colonies held at Copenhagen University, or from orphaned fragments of these colonies that rear sexuals in the absence of queens (Peacock *et al.*, 1955). Such colony fragments contained at least 50 workers, and young brood and eggs in a ratio of approximately five first-instar larvae or eggs to one worker. It takes approximately 6 weeks for eggs to develop into sexual imagoes (Peacock & Baxter, 1950). Sexual pupae can be distinguished into males and females one week before eclosion. The pupae were separated according to gender and housed in small plastic containers with an equal number of workers from the same colony, twice as many worker pupae, and five to ten times as many large larvae and eggs. Young brood was added because adult queens feed on larval secretions (Børgesen, 1989; Børgesen & Jensen, 1995). Pharaoh's ants are small and fragile; ants and brood were transferred using a thin moist paintbrush instead of tweezers.

All ants were kept at a constant temperature (25 °C) and constant relative humidity (30%), and were maintained under an LD 12 : 12 h photoperiod. To prevent escapes, the boxes containing the ants were coated with insect glue on the upper 1–2 cm of the inner walls, and placed on a shelf resting in a large dish filled with paraffin oil. The ants were fed with boiled chicken liver, boiled egg yolk, frozen *Drosophila* fruit flies, crushed almonds and crumbled cake, three times a week. Water was provided through moist cotton in a small plastic dish.

Induction of mating

Queens are receptive from 48–60 h after eclosion until the age of 14 weeks (Petersen & Buschinger, 1971). Depending on their mating status, males live only 3–8 weeks (Peacock & Baxter, 1950), but very young males frequently fail to inseminate the queens that they copulate with because their sperm is not fully mature

(Petersen & Buschinger, 1971). To obtain copulating pairs for morphological study, imagoes aged 16–18 days old were used. Mating was induced in a clean plastic container coated with glue on the upper 1–2 cm of the inner walls. The virgin queens to be mated were placed in groups of four or more individuals in the container together with some large larvae, and were left standing undisturbed for at least 10 min. The presence of workers in this container was avoided because they usually attack foreign males. Males were transferred one by one into the container with the virgin queens. Mating events usually started within seconds after introduction of the male. Because copulations last only 30–60 s, mating pairs were picked out of the container quickly with the moist paintbrush and transferred to the frozen surface of a cooling element. Sitting on top of this frozen surface, the mating pairs were immobilized immediately, allowing handling, and preparing for further histological study. Nine mating pairs were obtained by this method.

Histology

The ants were dissected in Ringer-Jolly solution, taking care not to separate the two gasters. The coupled gasters were fixed in glutaraldehyde, postfixed in osmiumtetroxide, dehydrated in a graded acetone series, embedded in araldite and sectioned with a Reichert Ultracut E microtome (Reichert-Jung, Wien, Austria). We stained the semithin sections (1 μm) with a methylene blue/thionine solution.

Multiple mating

To investigate the number of times a male can mate, individual virgin males were transferred to a box containing four virgin queens ($n = 20$), with a ratio of one male to 3–4 queens being reported for natural colonies (Peacock, 1951). Again, there were no workers present in these boxes. After the male died (usually within 24 h after his introduction), the insemination status of all queens was checked by dissection of the spermatheca. Because two of these males inseminated all four queens presented to them, the experiment was repeated with sets of eight virgin queens ($n = 8$).

Results

Figure 1 shows a longitudinal section through the gasters of a *M. pharaonis* pair in copula. Parts of the female genital tract can be identified: the common oviduct (approximately 500 μm long) containing the spermatophore, the spermatheca connected to the oviduct via the spermathecal duct (arrow), and the bursa copulatrix. The bursa copulatrix is a dorsal pouch-shaped expansion of the oviduct, lined with a very thick unsclerotized cuticle, secreted by the bursal epithelium (Fig. 2).

The oviduct of all nine females was filled with a cluster of spermatozooids followed by a compact mass of secretion (Fig. 1) representing the spermatophore. The sperm had yet to be transferred to the spermatheca via the

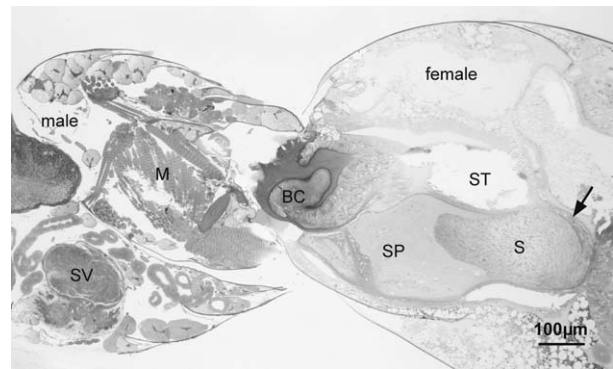


Fig. 1. Longitudinal section through the gasters of a *Monomorium pharaonis* pair in copula. On the right is the female abdomen, dorsal side up. The male (on the left, ventral side up) places his spermatophore (SP) inside her oviduct, with the mass of spermatozooids (S) first. The sperm is transferred to the spermatheca (ST) via the spermathecal duct (arrow). The male has seminal vesicles (SV) still filled with numerous sperm. BC, bursa copulatrix; M, muscles of the male genital capsule.

spermathecal duct. The male seminal vesicles still contained numerous sperm (Fig. 1).

Sections showed that the male genital appendices bear sharp teeth (Fig. 2, arrow), having a grip on an extension of soft, unsclerotized bursa cuticle hanging down like a septum from the ceiling of the bursa copulatrix. Close analysis of a dissected genital capsule under a dissecting microscope revealed that these teeth are situated on the ventral side of the two claspers or penis valves.

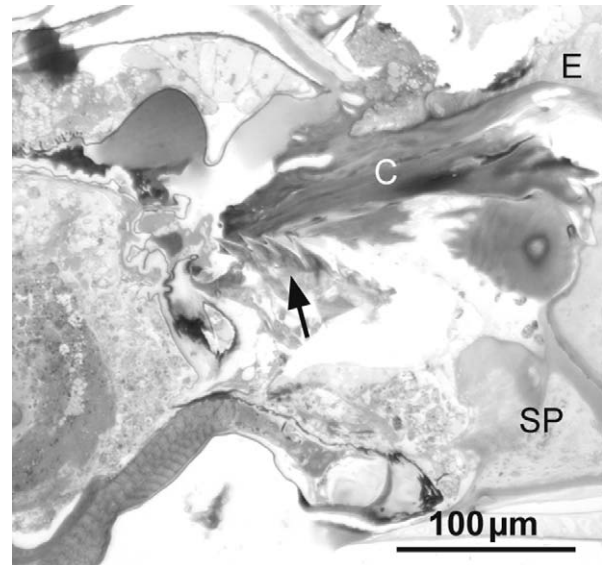


Fig. 2. Detail of a longitudinal section through the gasters of a *Monomorium pharaonis* pair in copula. The orientation of the specimens is the same as shown in Fig. 1. The female bursa copulatrix is a dorsal pouch formed by the most distal part of the oviduct. It consists of an outer epithelium (E), continuous with the oviduct, lined with a thick cuticle (C). The male genital appendices bear sharp teeth (arrow) that hold the bursal cuticle. SP, spermatophore.

The results of the experiment on male mating frequency are summarized in Table 1. In 11 cases, no successful insemination occurred. These cases were discarded because the males were not appropriate for mating during the experiment. Of the 17 remaining cases where the males successfully mated at least once, most males inseminated two females (eight cases out of 17), some were able to inseminate a maximum of four queens (three cases out of 17).

Discussion

Similar to *C. vidua* and *Diacamma* sp. males, *M. pharaonis* males transfer their sperm in a spermatophore. In insects, including the three ant species mentioned above, the mucous substances forming the spermatophore are secreted by the male accessory glands (Snodgrass, 1935; Chapman, 1998). Hung & Vinson (1975) found accessory glands in the males of 12 ant species belonging to five subfamilies, and Mikheyev (2003) confirmed the presence of accessory glands in 16 species from seven attine genera (Myrmicinae). Therefore, it is hypothesized that spermatophores are widespread among the Formicidae. Spermatophores inserted directly inside the female body represent a transitional stage between spermatophores that are deposited by the male and subsequently taken up by the female (as in apterygote insects) and the transfer of sperm directly into the spermatheca as in some honeybees (*Apis florea*; Koeniger *et al.*, 1989; *Apis andreniformis*; Koeniger *et al.*, 2000). In addition to this mechanical function of the spermatophore, other functions are possible. For example, Mikheyev (2003) discovered fatty acids transferred from the male accessory glands to the female of *Solenopsis invicta*, which are responsible for re-mating inhibition in the bumblebee *Bombus terrestris* (Baer *et al.*, 2001). Spermatophores could also function as a mating plug by blocking the female gonopore, preventing insemination by other males. To establish the exact function of spermatophores in ants will require further research.

Early drawings of male genitalia of Hymenoptera (Snodgrass, 1941) show that the serrated edges of the penis valves are no exception in male ants. The position of these teeth during copulation in *M. pharaonis*, in close

contact with the inner lining of the female genital tract, provides an indication on their function. The male probably uses them to attach himself to the female during copulation. The thickened cuticle of the corresponding part of the female genital tract indicates that this male adaptation might inflict harm on the female during copulation. Damaging mating tactics are not rare in insects. Injurious genital spines are known in beetles (Crudgington & Siva-Jothy, 2000) and dung flies (Blanckenhorn *et al.*, 2002; Mühlhäuser & Blanckenhorn, 2002). The damaging character of these traits usually arises as a negative pleiotropic side-effect of an adaptation that provides males with a reproductive advantage in another context (Morrow *et al.*, 2003). In a few cases, the harm inflicted is adaptive for the male, when the females respond to the harm by reducing their propensity to re-mate and/or elevating their current reproductive rate. In social insects, the male–female conflict over female mating frequency is an important issue due to the effects of mating frequency on colony genetic structure. A synopsis on queen–male conflict over mating and insemination is provided in a review on conflicts and alliances in insect families by Sundström & Boomsma (2001). Further research will reveal if the serrated penis valves help male ants in inhibiting polyandry.

The results of the present mating experiment are concordant with those obtained by Petersen & Buschinger (1971) indicating that, after more than 30 years in laboratory conditions, males of the same stock can still inseminate multiple females.

It might be argued that the current mating system of *M. pharaonis* (lack of a mating flight; males mating with multiple females inside the nest) is a strongly derived condition, caused by the insular nature of appropriate habitats (permanently heated buildings) in the recently colonized temperate regions, and that the results of the present study are not illustrative for 'wild' species. Petersen & Buschinger (1971) expressed their concern on this matter already in the conclusion of their study on the mating behaviour of the pharaoh's ant. In the present study, it is argued that the observed mating system is already present probably in the 'wild' populations of the pharaoh's ant. First, mixed mating strategies are reported for other ants, including the argentine ant *Linepithema humile* Mayr, another cosmopolitan pest ant (Passera & Keller, 1994). Males of *L. humile* have two alternative mating strategies: (i) when females are available in the maternal colony, males will mate inside the nest with those females and (ii) if females are not available in their own colony, males will disperse and enter foreign colonies to find females. Similarly, mating inside the nest with related females might have existed in *M. pharaonis* long before the colonization of new habitats, and be the persistent mating strategy in an environment in which it is impossible for individual alates to locate conspecific colonies. Moreover, the sudden switch from a strategy of total dispersal to a strategy of zero dispersal, coupled with the discarding of relatedness barriers between sexual partners and the increased burden of inbreeding, does not sound very realistic. Although it is very speculative, it is

Table 1. Results of the mating experiment: to investigate how many times males of *M. pharaonis* mate, single virgin males were presented with sets of four or eight virgin females.

	Experiment with four virgin queens ($n = 20$)	Experiment with eight virgin queens ($n = 8$)
No queens inseminated	10	1
One queen inseminated	3	2
Two queens inseminated	4	4
Three queens inseminated	1	0
Four queens inseminated	2	1
More than four queens inseminated	0	0

probable that 'wild' populations (not living inside permanently heated buildings) of *M. pharaonis*, which to our knowledge have not been reported in the past few decades, may show a mixed mating strategy with low dispersal.

In conclusion, despite the basic differences between *C. vidua*, *Diacamma* sp. and *M. pharaonis* in the number of matings, sex ratio and mating syndrome, these species show pronounced similarities with regard to sperm transfer, including the production of a spermatophore and the use of specialized genitalia to connect to the female. The present study is only the starting point for a deeper analysis of ant mating systems. More species will need to be studied in the light of sexual selection and male–female conflict before any conclusions can be made with respect to the selective forces that shaped these mating systems.

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