

Timing of sperm transfer in *Diacamma pallidum*

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Abstract. *Diacamma* species (Hymenoptera, Formicidae) differ from other ants by the extremely long duration of copulation. By using histological sections through mating pairs of *Diacamma pallidum* (F. Smith), it is demonstrated that the transfer of sperm to the female genital tract only takes 2 min and is completed quickly after the onset of copulation, although the male and female will remain connected for many hours. Next to the two traditional hypotheses of mate guarding and mate manipulation commonly invoked to explain prolonged copulations, a new hypothesis is proposed linked to the interference of the nestmate workers with the mating pair, and suggestions for further research are given.

Key words. *Diacamma pallidum*, Formicidae, mate guarding, prolonged copulation, spermatophore.

Introduction

Males gain from monopolizing females because it ensures exclusive paternity over a female's offspring. Insect males have evolved a variety of methods to prevent competitors from fertilizing their mates' gametes, including mate guarding (e.g. the dragonfly *Sympetrum parvulum*; Uéda, 1979), transfer of a mating plug (e.g. the bumblebee *Bombus terrestris*; Baer *et al.*, 2001), sperm displacement by removal of competitor sperm from the female genital tract (e.g. the dung fly *Scatophaga stercoraria*, Parker, 1970; the damselfly *Calopteryx maculata*, Waage, 1979), or transfer of inhibitory substances in seminal fluids in *Drosophila melanogaster* (Chen *et al.*, 1988; Wolfner, 1997). These activities require time and may sometimes lead to prolonged copulations. Prolonged refers to the investment by the male of added time in mating, above and beyond that needed for insemination of the female. According to Dickinson (1997), another cause of prolonged copulations is that the pair in copula is safer from predators (i.e. if pairs can detect predators sooner, if they confuse predators, or if there is augmented chemical defense when paired); the male stimulating the female mechanically to reduce her sexual receptivity (receptivity-reduction hypothesis; Chen, 1984) or to ensure fertilization (sexual stimulation hypothesis; Eberhard, 1985) can also cause prolonged matings. For example, mechanical stimulation by the male genital plug triggers ovarian activation in queens of the stingless bee *Melipona quadrifasciata* (Melo *et al.*, 2001).

In species of the queenless ant genus *Diacamma*, copulation can last for 2 days (Fukumoto *et al.*, 1989; Nakata *et al.*, 1998), which is remarkably lengthy compared with other ants (Table 1). *Diacamma* is one of the queenless ant genera belonging to the subfamily Ponerinae. Queens do not exist but gamergates (i.e. mated workers laying fertilized eggs), perform the reproductive tasks (Peeters & Crewe, 1984). Each *Diacamma* colony is headed by a single gamergate, which subjects every freshly eclosed worker to a mutilation that obliterates her mating ability (Fukumoto *et al.*, 1989; Peeters & Higashi, 1989; Sommer *et al.*, 1993; Kikuta & Tsuji, 1999). When a colony loses its gamergate, a young un mutilated worker will mate and replace her predecessor. Fukumoto *et al.* (1989) and Nakata *et al.* (1998) describe the mating sequence of the Japanese *D. rugosum* in detail: the young receptive worker leaves the nest in the evening, approximately 10 days after eclosing, and releases a sex pheromone from her metatibial glands to attract foreign males (Nakata *et al.*, 1998). Shortly after the beginning of copulation, the worker returns inside the nest with the male still attached to her abdomen by his genitalia. After a further period, the worker bends around and starts biting the male, and her nestmates sever his wings, legs and head, resulting in his death. Even then the male abdomen remains attached to the worker for several hours to 2 days, until the worker finally succeeds in detaching it. Male ants typically have an elaborate genital architecture, which functions in clasping the female's cloaca during copulation and allows the male to remain attached to his partner by his genitalia alone, even if she is in flight (Bourke & Franks, 1995). Allard *et al.* (2002) analysed the mechanical aspects of this strong connection in *Diacamma* sp. from Japan by using histological sections through copulating pairs.

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Table 1. Copula duration in various ant species.

Species	Copula duration	Reference
<i>Meranoplus peringueyi</i>	25 s (average)	Robertson & Villet (1989)
<i>Monomorium pharaonis</i>	40–60 s, maximum of 4 min	Petersen & Buschinger (1971)
<i>Leptothorax acervorum</i>	1 min	Franks <i>et al.</i> (1991)
<i>Crematogaster</i> sp.	1 min 19 s (average)	Robertson & Villet (1989)
<i>Formica (Formica) yessensis</i>	3–5 min	Higashi (1983)
<i>Carebara vidua</i>	4 min	Robertson & Villet (1989)
<i>Acromyrmex versicolor</i>	4–5 min	Reichardt & Wheeler (1996)
<i>Iridomyrmex humilis</i>	6 min	Keller & Passera (1992)
<i>Dinoponera quadricaps</i>	30 ± 18 min (<i>n</i> = 9)	Monnin & Peeters (1998)
<i>Eciton hamatum</i>	10 h (<i>n</i> = 1)	Schneirla (1971)
<i>Eciton</i> sp.	10 h	Schneirla (1949); Rettenmeyer (1963)
<i>Diacamma</i> sp. from Japan	Several hours to 2 days 20–24 h	Fukumoto <i>et al.</i> (1989) Nakata <i>et al.</i> (1998)

In the present study, whether the total duration of copulation in *Diacamma pallidum* (F. Smith) exceeds the time necessary for successful sperm transfer is tested, and attempt is made to formulate a hypothesis for this prolonged mating. For this purpose, virgin males and receptive workers are reared, allowed to copulate for different times, and fixed in copula, after which they are sectioned longitudinally to visualize the progress of sperm transfer.

Materials and methods

Ten colonies of *D. pallidum* were excavated from the forest soil around the Ulu Gombak Field Station, Malaysia, during August 2002, and transferred to Belgium. Only five of them contained a gamergate and these five colonies were used as a source of female pupae. The other five colonies were used for the rearing of males.

Maintenance of colonies

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

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Rearing of virgin males and females

To obtain virgin males, ten subcolonies of ten mutilated workers were created from the orphan colonies. These workers were laying eggs because a gamergate was absent. Approximately 2 months later, adult males started emerging. To obtain virgin unmutilated females, ten subcolonies consisting of six mutilated workers and one cocoon were created from the non-orphan colonies. The callows eclosing from these cocoons were not mutilated because a gamergate was absent, and were therefore able to mate.

Induction of mating

Mating experiments were carried out at approximately 18.00 h, corresponding to the period of sexual activity observed by Fukumoto *et al.* (1989) and Nakata *et al.* (1998). Small (5 × 5 × 4 cm) translucent plastic boxes with a translucent lid were used as the experimental arena. A 10-day-old virgin female was carefully placed in the box with a pair of forceps and left undisturbed for a few minutes and then a single male was added. Copulation started usually within 5 min of the introduction of the male. Mating was induced in ten pairs of ants. These pairs were killed after different times to visualize the progress of sperm transfer.

Dissections and histology

The ants were killed by placing them in the freezer for a few minutes. Their gasters were dissected in Ringer–Jolly solution under a dissecting microscope. The connected abdominal tips of the copulating individuals, or the female abdomen alone (after spontaneous interruption of copulation), 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 longitudinally using a Reichert Ultracut E microtome (Reichert, Austria). Semithin sections (1 µm) were stained with a methylene blue/thionine solution.

Results

Approximately 15 min after the start of copulation, the oviduct was filled already with a spermatophore (Fig. 1a). The spermatophore consisted of a gelatinous mass, produced by the male accessory glands (Allard *et al.*, 2002), and contained the sperm. The sperm mass encapsulated at the top of the spermatophore had reached already its final position in the oviduct, underneath the spermatheca (the associated gland of the spermatheca is visible on the section; Fig. 1a). One hour after the start of copulation, the spermathecal duct showed a dark filling, which means that the transfer of sperm through this duct, into the spermathecal reservoir, had started already (Fig. 1b). Approximately 1 h and 45 min after the

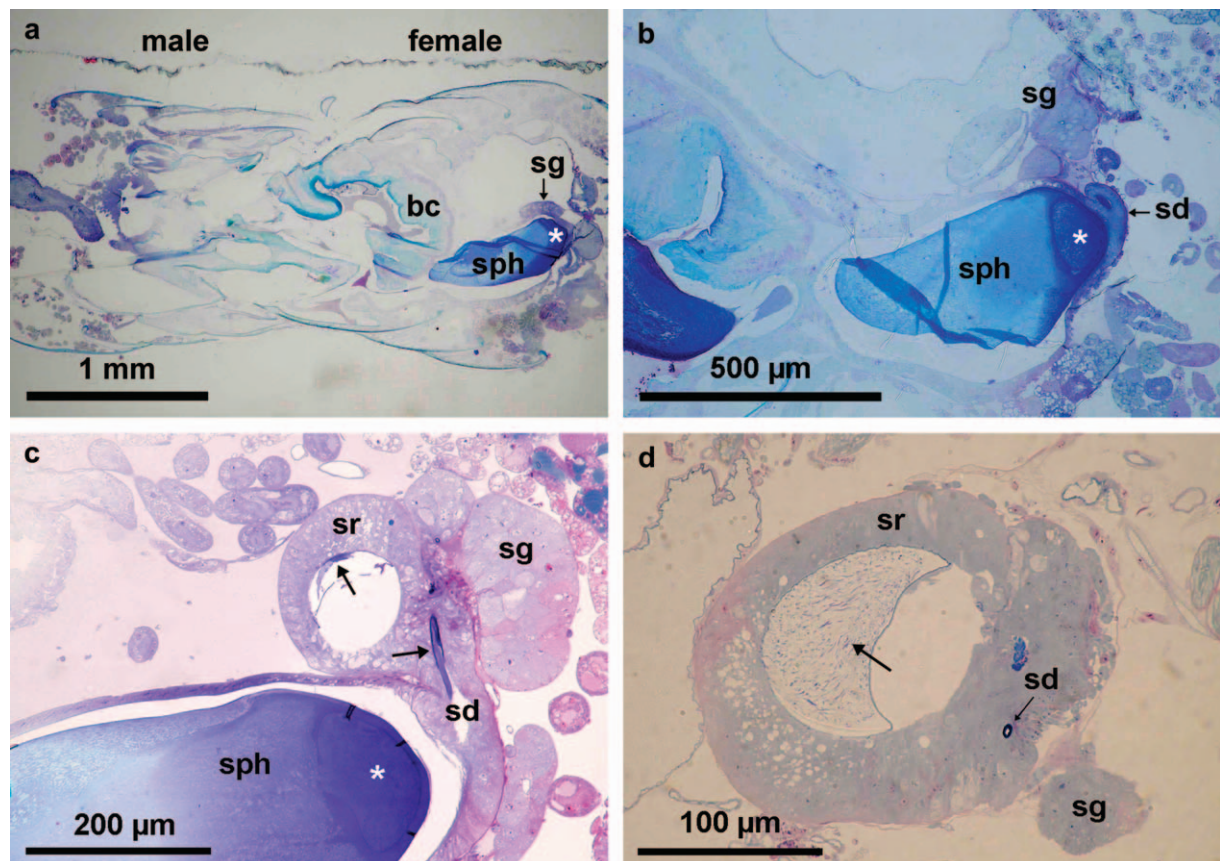


Fig. 1. Longitudinal section through (a) abdomen of mating pair, 15 min after start of copulation; (b) genital tract of mating female, 1 h after start of copulation; (c) genital tract of mating female, 1 h and 45 min after start of copulation; (d) spermatheca of mating female, 22 h after start of copulation. bc, Bursa copulatrix; sd, spermathecal duct; sg, spermathecal gland; sph, spermatophore; sr, spermathecal reservoir.

start of copulation, the sections revealed the presence of the first sperm inside the spermathecal reservoir (Fig. 1c), and 22 h after the start of copulation, the spermatheca appeared to be filled with sperm (Fig. 1d).

After the female had been freed from the male, she expelled the emptied spermatophore. Figure 2(a) shows the female genital tract, freed from the male after approximately 15 h of copulation. The spermatophore was still present inside the oviduct but had been sliding down slightly, away from the spermatheca. The sperm capsule was empty, showing an aperture at the top. Sperm was present inside the spermathecal reservoir. Sections through another female, 23 h after the start of copulation (and freed from the male at an unknown point in time), showed the same situation (Fig. 2b). Thirty hours after the start of copulation (and also freed from the male at an unknown point in time), the spermatophore had completely disappeared (Fig. 2c). The spermathecal reservoir of this specimen seemed to hold only a small amount of sperm, an artefact due probably to compression of the spermathecal content during the sectioning process. The same artefact appeared on sections through a female 1 day after the end of copulation (Fig. 2d).

Discussion

Does the total duration of copulation in *D. pallidum* far exceed the time necessary for successful sperm transfer? The spermatophore reaches the appropriate position after less than 15 min of copulation. Sperm cells have already reached the spermathecal reservoir after 1 h and 45 min. The amount of sperm present inside the spermatheca appears to be limited, whereas a great concentration of sperm is still visible in the spermatophore. Although proving very useful for tracking the process of sperm transfer during and after copulation, histological sectioning appears to have limited use for estimating the amount of sperm inside the spermatheca. The spermathecal reservoir consists of a spherical epithelium lined on the inner side by a tough cuticle. Consequently, the cuticle is almost always torn from the epithelium during sectioning. The initially spherical cuticle is compressed, like a deflating football, along with its content. Full spermathecae therefore rarely show up as such on histological sections. When copulation ends, the spermatophore has been emptied from its sperm cells.

A study of sperm transfer in the pharaoh's ant *Monomorium pharaonis* reveals that males need just a few seconds to transfer

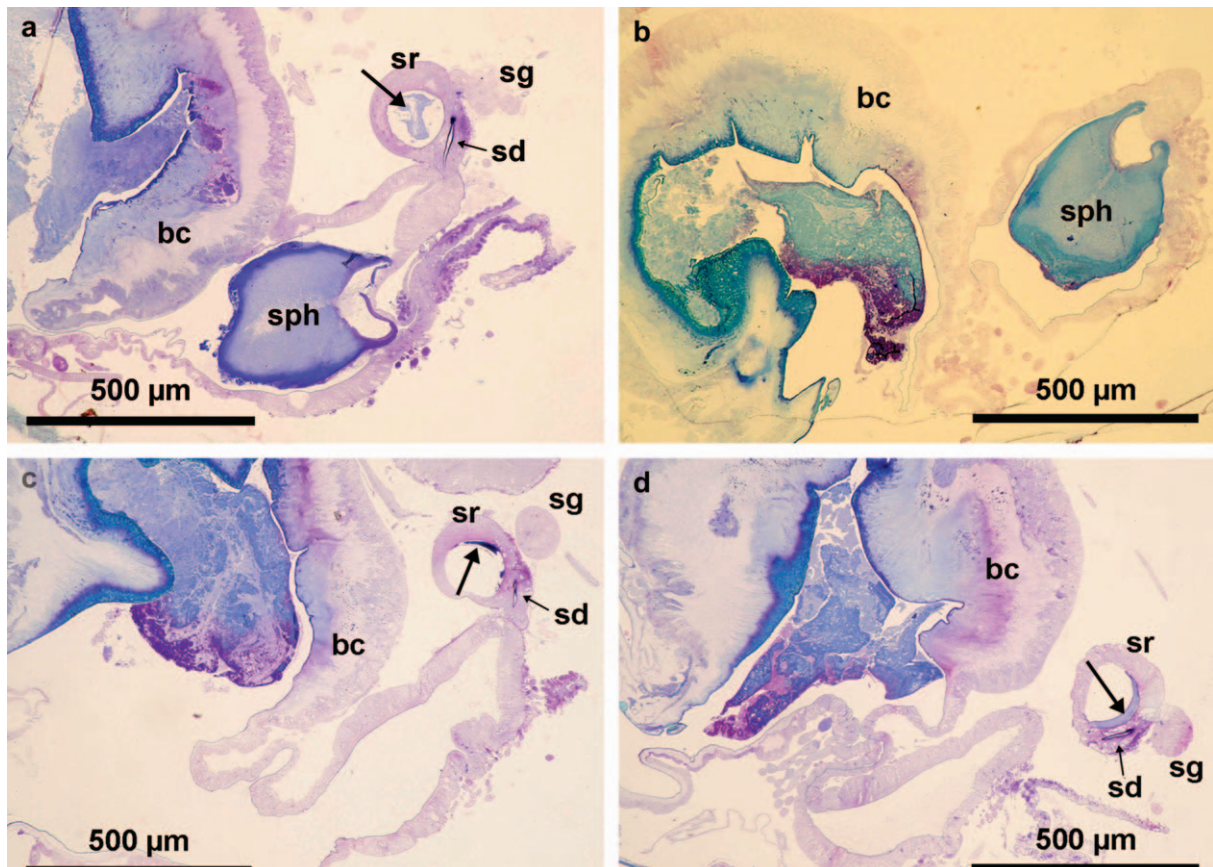


Fig. 2. Longitudinal sections through genital tract of females after the end of copulation: (a) immediately after a 15-h long copulation; (b) 23 h after the onset of copulation; (c) 30 h after the onset of copulation; (d) 24 h after the end of copulation. Arrows indicate sperm cells present in the female genital tract; an asterisk indicates a sperm packet in the spermatophore. bc, Bursa copulatrix; sd, spermathecal duct; sg, spermathecal gland; sph, spermatophore; sr, spermathecal reservoir.

a spermatophore, after which they release the female (Allard *et al.*, 2006). In *Carebara vidua*, copulation lasts an average of 4 min, and sperm is shunted from the spermatophore to the spermatheca after copulation has ended (Robertson, 1995). The active role of the male in the sperm transfer process is thus finished after transfer of the spermatophore. Sperm transfer from the spermatophore to the spermathecal reservoir then depends on the female, and is probably achieved through haemolymph pressure (Robertson, 1995). It therefore appears that the length of copulation in *Diacamma* is extremely excessive, and that it must have some other function(s) than simple transfer of sperm.

Several hypotheses on the cause of the prolonged copulation can readily be eliminated. It is doubtful that *Diacamma* males are seeking protection from predators by prolonging copulation because the pair enters the nest soon after the start of copulation where predators are absent and where the male is torn to pieces by the workers. They are not trying to remove sperm from a predecessor because *Diacamma* females mate only once (Nakata *et al.*, 1998). Physical thwarting of competitors by the male appears to be equally unlikely because male competitors are not present

inside the nest. The two remaining hypotheses (i.e. the mate-guarding hypothesis and the sexual stimulation hypothesis) appear to be the most appropriate to explain prolonged copulation in *Diacamma*. Copulation is not linked to ovarian activity (i.e. virgin workers can lay eggs when the gamergate is removed) but could lower the female's propensity to remate. Also, the long copulation might simply overspan the period during which the worker is sexually receptive. Testing this would require artificial interruption of copulation and subsequent testing of the female's receptiveness towards new males. This would reveal whether females mate singly because of preference, or because the males enforce it.

This suicidal attachment of male ants to their partner has also been described in *Pogonomyrmex* (males leave their genitalia stuck to the female's cloaca after mating; Hölldobler, 1976) and *Dinoponera quadriciceps* (males are dragged in copula inside the nest, where the female succeeds in cutting off the end of the male's abdomen after 1–2 min, leaving part of the male sexual apparatus attached to the female's genital tract for ≥ 30 min; Monnin & Peeters, 1998). These extreme forms of mate guarding can be explained by the rarity of

mating opportunities for ant males, such that complete investment in one mating is advantageous.

Finally, the interference of the workers with the mating pair might contribute to the strength and longevity of the connection between male and female. Copulation with flightless females inside the nest is a derived condition in ants. Most ant species show nuptial flights during which males and females meet in the air and on vegetation some distance from their natal nest; copulation in *Diacamma* takes place inside the female's natal nest, where interference by workers is important (Fukumoto *et al.*, 1989; D. Allard, unpublished observations). Testing the influence of workers on copula duration would require comparing copula duration in presence and absence of workers. Other genera where mating is with foreign males inside the nest include *Ophthalmopone*, *Rhytidoponera*, *Megaponera* (Peeters, 1991) and *Linepithema humile* (= *Iridomyrmex humilis*) (Keller & Passera, 1992, 1993; Passera & Keller, 1994). In army ants, workers appear even to select which males gain access to the queen. Army ant males have developed similar channels of communication as the queens use, apparently as a result of mate choice via the workers (Franks & Hölldobler, 1987).

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