

Mating Biology of the Leaf-Cutting Ants *Atta colombica* and *A. cephalotes*

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ABSTRACT Copulation behavior has often been shaped by sexually selected sperm competition or cryptic female choice. However, manipulation of previously deposited ejaculates is unknown in the social Hymenoptera and the degree to which sperm competes after insemination or is actively selected by females has remained ambiguous. We studied the mating process in the leaf-cutting ants *Atta colombica* and *A. cephalotes*, which belong to one of the few derived social insect lineages where obligate multiple mating has evolved. As copulations often occur at night and in remote places, direct observations were impossible, so we had to reconstruct the sequential copulation events by morphological analysis of the male and female genitalia and by tracking the process of sperm transfer and sperm storage. We show that *Atta* male genitalia have two external rows of spiny teeth, which fit into a specialized pouch organ in the female sexual tract. Reconstruction of the sperm storage process indicated that sperm is transferred to the spermatheca during or immediately after ejaculation and without being mixed with sperm and seminal fluids from other males. A convergent mechanism of direct sperm transfer to the spermatheca of queens is known from two species of dwarf honeybees. Direct sperm transfer may restrict female control over the sperm storage process and the number of males that contribute to the stored sperm. *J. Morphol.* 267:1165–1171, 2006.

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Copulation behavior initially evolved under natural selection as an act of mutual benefit to both sexes involving the transfer of sperm (Simmons, 2001). However, the same process also quickly became a battlefield for sexual selection, as males evolved traits to increase their reproductive success and females evolved counter-adaptations to restrict male manipulations (Eberhard, 1985, 1996; Birkhead and Møller, 1998). The transfer of sperm and seminal fluids is a complicated process, as ejaculates contain a wide range of proteins influencing female behavior and physiology (Simmons, 2001; Kubli, 2003; Baer 2003, 2005). Consequently, the study of ejaculate transfer can provide interesting insights into the evolutionary history of sexual conflict and has therefore been studied intensively in many organisms.

Compared to most well-studied animals, the study of copulation and sexual selection in social insects is still in its infancy. Recent reviews (Baer 2003, 2005; Boomsma et al., 2005) have established that the social Hymenoptera are unusual in that they neither have sophisticated courtship behaviors, nor promiscuity in the usual sense. Females (queens) mate only during a few hours early in adult life (typically during a single nuptial flight) (Baer, 2003). Mating is therefore not part of normal social life and there is only a single brief and “presocial” time window during which sexual selection can have a direct effect or set the stage for later expressions of conflict between the sexes.

Copulation behavior has been investigated in only a few species of social insects and we have only limited knowledge about the evolution of social insect mating systems (general reviews in Boomsma and Ratnieks, 1996; Strassmann, 2001; Boomsma et al., 2005). The information available indicates that mating behavior can be quite spectacular. In the honeybee, for example, copulations take place on the wing during mass swarming (Winston, 1991), last only a couple of seconds (Koeniger and Koeniger, 1991), and are suicidal for males because the protrusion of their endophallus is irreversible and lethal (Koeniger and Koeniger, 1991). Honeybee queens mate with many males in quick succession, thereby acquiring up to 300 million spermatozoa in their lateral oviducts (see Baer, 2005, and references therein). The sperm storage process starts after the mating flight and takes about 40 h (Woyke, 1983). Only a small proportion, ~2.5% of the sperm, equivalent to a single ejaculate, finally ends up in the spermatheca.

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Copulation dynamics are quite different in bumblebees (Baer, 2003), which copulate for 9–180 min, depending on the species (Brown and Baer, 2005). In *Bombus terrestris* sperm is ejaculated into the bursa copulatrix shortly after the onset of copulation and sperm storage takes about 90 min and starts immediately after ejaculation (Duvoisin et al., 1999). Bumblebee males of at least three species transfer a mating plug to the female during copulation (Brown and Baer, 2005), which reduces her willingness to remate in *B. terrestris* (Sauter et al., 2001; Baer et al., 2001). The transfer of male accessory gland compounds as spermatophores is also known from the ants *Carebara vidua* (Robertson, 1995) and *Diacamma sp.* (Allard et al., 2002). There is further evidence for the transfer of mating plugs in fire ants (Mikheyev, 2003), but with the exception of *B. terrestris* the functional details of these mating plugs and spermatophores are unknown (Baer, 2003, 2005; Boomsma et al., 2005). The *Apis* honeybees also have mating plugs, but these no longer function as inhibitors of further mating attempts (Baer, 2005). Suicidal male mating as found in honeybees is also known from the ponerine ants *Dinoponera quadriceps* (Monnin and Peeters, 1998) and *Diacamma* species (Allard et al., 2002). Finally, in the ant *Hypoponera opacior*, males copulate with nonclosed queens for up to 40 h by inserting their genitalia through the cocoons from which virgin queens are about to hatch (Foitzik et al., 2002).

Here we reconstruct the copulation process in the leaf-cutting ants *Atta colombica* and *A. cephalotes*. *Atta* species have been expected to have a series of unusual adaptations for mating and sperm transfer. First, they are among the most fertile animals known (Weber, 1972), with single queens storing several hundred million sperm (Fjerdingstad and Boomsma, 1998; Baer et al., 2006). Second, the observation that the average number of queen matings in *Atta* is lower than in its sister genus *Acromyrmex*, in spite of much larger colony sizes and sperm stores (Fjerdingstad and Boomsma, 1998; Villesen et al., 1999), suggests that selection may have limited the number of males that contribute to the stored sperm. Recent work has shown that the number of sperm stored and the number of mates contributing to the stored sperm both incur immunity costs during colony founding for *A. colombica* queens (Baer et al., 2006), which may constrain the number of matings. However, this implies that queens are unable to reject excess sperm or ejaculates. Copulations of *Atta* species cannot be observed directly because they occur mostly at night and probably high up in the air (Weber, 1972). However, mass swarming during take-off and landing allowed us to collect both mature virgin sexuals and newly mated queens of *A. colombica* and *A. cephalotes*, and to reconstruct the events during copulation from a series of dissections.

MATERIALS AND METHODS

Reproductives of the leaf-cutting ants *Atta colombica* and *A. cephalotes* were collected in Gamboa (Republic of Panama) at the start of the rainy season (April–June) in the years 2001–2004. Sexuals were collected in four different ways: 1) Virgin queens and males were excavated from mature colonies during their emergence from nest entrance holes just before nuptial flights. 2) Newly inseminated, winged queens were collected during nuptial flights under streetlights in Gamboa, where they became temporarily trapped (Moser, 1967) and fell to the ground. 3) Freshly inseminated, dealated queens were collected while they were wandering around or digging their nest burrows. 4) Queens were excavated from their fungus chambers 1 day, 14 days, and 1 year after their mating flight. In order to stop the sperm storage process, queens collected during and shortly after the mating flight were immediately freeze-killed and kept at -20°C for future inspection.

Dissections were performed with a Leica stereomicroscope at magnifications between $12.5\times$ and $64\times$ and pictures of reproductive tracts were taken with a Canon EOS D30 digital camera. These pictures were transferred to a Macintosh computer and size measurements were taken using the public domain Image 1.62 program (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image>).

In all insects investigated so far, individual males are characterized by their own typical sperm length (Ward, 1998; Morrow and Gage, 2001; Baer et al., 2003). Here we show that this is also true for *Atta colombica* males. We used this phenotypic character to test for sperm clumping within the spermatheca of newly inseminated females by comparing distributions of sperm length across samples from a single spermatheca. Sperm length was estimated using a standard technique described previously (Baer et al., 2003; Baer and Boomsma, 2004). We collected sperm samples by puncturing the sperm-containing organs (spermathecae and seminal vesicles) with a needle. A small subsample of out-flowing sperm was picked up with a glass capillary and smeared over the surface of a microscope slide, where it was allowed to air dry. To measure the length of sperm we used a Leitz differential interference contrast (DIC) microscope connected to a digital camera. Slides were checked at $400\times$ magnification and digital DIC microscope images of 10 nondamaged sperm were taken for each sperm smear. Sperm length was subsequently measured by analyzing the images with the NIH image 1.62 software.

To compare sperm length between males, we excavated 20 mature males per colony from five different colonies of *Atta colombica*. Sperm samples were always taken at the distal end of the right accessory testis. We also tested for variation in sperm length within a male, using 12 males from five colonies, by collecting sperm samples from the ejaculatory section as well as from the dorsal and distal end of the sperm reservoir of each accessory testis. To test for the presence of sperm clumping within the spermatheca, we resampled sperm from each lobe of the spermatheca (see Fig. 1) six times, so that a total of 12 replicate sperm smears became available for each spermatheca. A total of 12 queens was used for this work. All of these queens were excavated 1 week after the mating flight. Statistics were done using SPSS 11 for Macintosh and all *P*-values are reported with two-tailed probabilities. Nested ANOVAS were manually programmed in the syntax.

RESULTS

Queens of *Atta colombica* and *A. cephalotes* have the same general morphology of their sexual tracts. The only difference was that sexuals of *A. cephalotes* were larger than those of *A. colombica*. We therefore begin by presenting a general overview of *Atta* reproductive organs. Large-scale nuptial flights and sufficient numbers of queens to

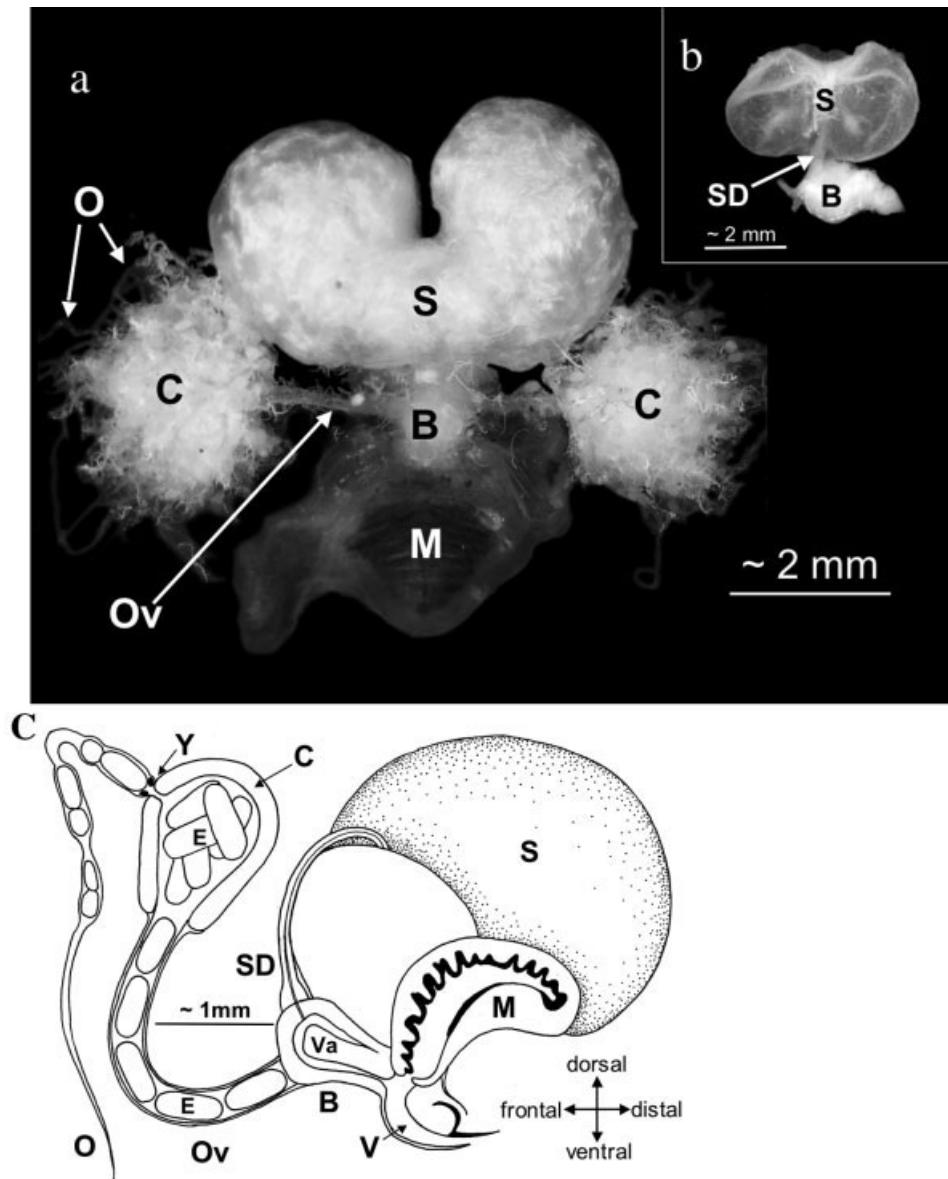


Fig. 1. **a:** Digital picture of the sexual tract of a newly inseminated *Atta cephalotes* queen. **b:** The bursa and the spermatheca of a winged virgin queen excavated from a colony before the mating flight. **c:** Cross section through the sexual tract of an established nest queen of *A. colombica*. Only a single ovariole is shown for simplification (in reality there are several hundreds). B, bursa copulatrix; C, calyx; E, eggs; M, mussel organ; O, ovarioles; Ov, lateral oviducts; S, spermatheca; SD, spermathecal duct; Va, valve; V, vagina; Y, yellow bodies.

study the sperm storage process were only available for *A. colombica*, so that the results reported in later sections are only based on data for that species.

Female Sexual Organs

A general overview of the queen's sexual tract in *Atta colombica* and *A. cephalotes* is given in Figure 1, based on dissections of more than 50 queens. The central part of a queen's sexual tract consists of the bursa copulatrix, a complex organ with an envelope

of muscular tissue, and a central muscular valve that divides it into several compartments (Fig. 1c). An extremely large spermatheca consisting of two spherical lobes is situated dorsally of the bursa copulatrix. In virgin queens the spermatheca is flat and looks like a noninflated balloon (Fig. 1b). A spermathecal duct connects the dorsal part of the spermatheca with the anterior part of the bursa copulatrix. There is no tissue that could function as a spermathecal pump along the spermathecal duct, as is found in honeybees (Bresslau, 1905). However, a rather peculiar structure is found at the dorsal end

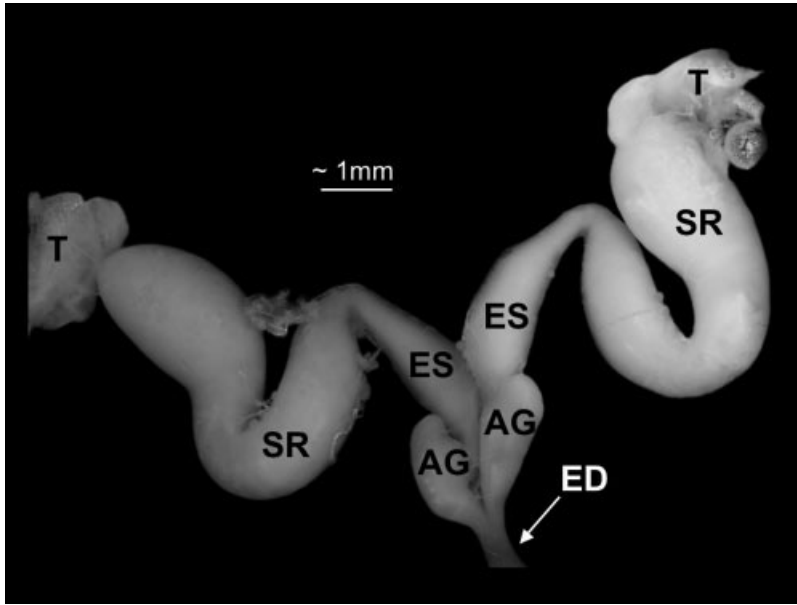


Fig. 2. The sexual organs of a mature *Atta colombica* male before the nuptial flight. AG, accessory glands; ES, ejaculatory sections; ED, ejaculatory duct; SR, sperm reservoir; T, testis.

of the bursa copulatrix, which we coined the “mussel organ.” This organ is a pouch with sclerotized lamellae that open into the vagina (Fig. 1c) between the bursa and the sting chamber. Each of the two oviducts connects to the frontal side of the bursa copulatrix with a calyx, a temporary reservoir, in which the hundreds of ovarioles release mature eggs and from where these eggs are transported one after another down the oviducts into the bursa copulatrix.

Male Sexual Organs

The male reproductive organs were found to be similar to those of other hymenopteran social insects (Hölldobler and Wilson, 1990; Baer, 2003; Boomsma et al., 2005), although several derived characteristics were detected that function as adaptations to multiple mating by both queens and males (see also Baer and Boomsma, 2004). Immature males that were excavated about 3 weeks before their mating flight had large testes but no sperm in the accessory testes, the typical sperm storage organ of mature social insect males. Mature *Atta* males collected during nuptial flights, however, had enlarged accessory testes that were densely packed with sperm, and reduced testes that no longer contained sperm (Fig. 2). This implies that *Atta* males do not produce sperm after sexual maturation, in agreement with what is known for almost all other ants (reviewed in Boomsma et al., 2005). However, in contrast to most other hymenopteran social insects, the accessory testes of leaf-cutting ants consist of two distinct structures (Fig. 2), an upper larger part, acting as a sperm reservoir, and a lower part, the ejaculatory section. Males of both *Atta* species also have accessory glands, but they are small compared to other attine ants (Baer and Boomsma, 2004). However,

they seem functional since dissections revealed that they contain a gelatinous sticky secretion very similar in consistence to what has been reported in bumblebees and fire ants. Furthermore, males have large complex external genitalia, with two conspicuous rows of sclerotized, backward bent teeth (Fig. 3).

Male decapitation induces sexual stimulation and sometimes results in ejaculation, which starts by the protrusion of the external genitalia. The two rows of sclerotized teeth start making “sawing” movements independently of each other, which implies that a male uses these structures to actively attach himself to the female during copulation: While the first row of teeth is hooked into the lamellar structure of the female mussel organ (Fig. 3), the second row can penetrate even deeper, so that these rows of teeth act as a connecting mechanism that promotes forward but prevents backward movements. The result of this “male sawing behavior” is a firm connection between the mating pair, which we simulated by dissecting the mussel organ and placing it over protruding male genitalia. This confirmed that the mussel organ perfectly fits the sclerotized male genitalia and that the connection is tight because it took some force to remove the mussel organ once the male teeth were successfully hooked into its lamellae. Further evidence that the mussel organ acts as an anchoring device for the male genitalia came from dissections of inseminated queens, where the central part of the lamellar structure showed signs of damage (Fig. 3) and where lines of black dots in the underlying tissue indicated melanization, as is typically found after wound healing in insects. These scar lines within the mussel organ match the inferred position of the rows of teeth of the male

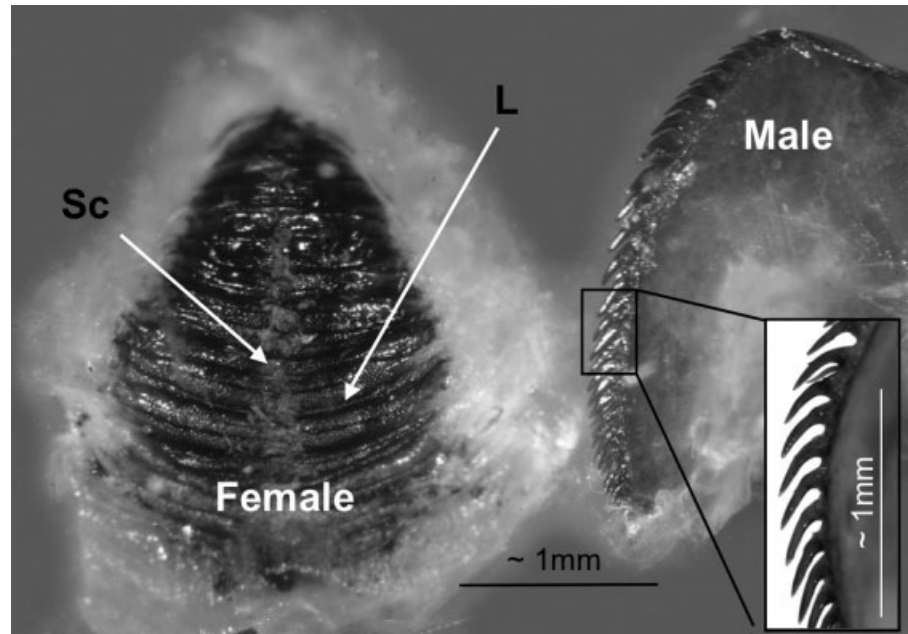


Fig. 3. The lamellar structure (L) of the mussel organ (left) of a newly inseminated queen of *Atta colombica* and the possible scarring damage (Sc) caused by the teeth of the male reproductive organs (white arrows). One of the two rows of spiny teeth from the male external genitalia is shown in the right-hand figure.

genitalia and were never observed in dissections of mussel organs of virgin queens.

After decapitation and protrusion of the male genitalia a small and tiny gelatinous mass appears at the tip of the sclerotized genitalia, followed by a massive amount of sperm that is forcefully expelled. Obviously, males transfer a small mating plug to the female, and the most likely origin of this mating plug is the accessory glands (see above). Our dissections also showed that sperm gets ejaculated from only one of the ejaculatory sections, although it is difficult to say whether this is also true for natural copulations. After ejaculation, sperm from the male sperm reservoir is transported to the empty ejaculatory section by contractions of the accessory testes and a second ejaculation can occur shortly afterwards, as described previously (Baer and Boomsma, 2004).

Sperm Transfer and Sperm Storage During Nuptial Flights in *Atta colombica*

All queens dissected during or after the mating flight had large amounts of sperm in their spermatheca, but neither the bursa nor the spermathecal duct were greatly enlarged or contained more than residual amounts of sperm. The lateral oviducts never contained any sperm. In queens excised and dissected 2 weeks after the mating flight, sperm was only detected in the spermatheca. Interestingly, not all queens had received equal amounts of sperm—out of the 10 mated queens investigated, one queen had a completely empty spermatheca and one was only partially inseminated because the spermathecal volume was substantially smaller compared to that of the other inseminated queens.

Nevertheless, all these queens had started a colony and were maintaining a small fungus garden.

Our analysis of sperm length revealed that spermatozoa are of similar morphology as found in other hymenopteran social insects, with an average length of $162.03 \pm 7.64 \mu\text{m}$ (mean \pm SD, $n = 100$). Sperm length can be used as a phenotypic male character in *Atta colombica*, because we found significant differences not only between males of different colonies (nested analysis of variance [ANOVA], $F_{4,95} = 23.431$; $P < 0.001$), but also among brother males of the same colony ($F_{95,900} = 17.192$, $P < 0.001$). Sperm length did not differ across samples from the same male, either between samples taken from the same accessory testis ($F_{5,285} = 1.322$, not significant [n.s.]), or between the left and the right accessory testis ($F_{4,5} = 0.435$, n.s.), although we found once more a significant difference in sperm length between males (nested ANOVA, $F_{4,4} = 22.96$, $P < 0.001$).

The length of stored sperm within a spermatheca showed significant differences between queens (nested ANOVA, $F_{6,6} = 27.765$, $P < 0.001$). More important, we also found significant differences in sperm length within samples of the same spermatheca ($F_{10,816} = 4.610$, $P < 0.001$), but not between the two spermathecal lobes ($F_{6,10} = 0.958$, n.s.). This indicates that sperm from different males has not become completely mixed after storage shortly after mating.

DISCUSSION

The striking similarities in a series of derived genital traits between *Atta colombica* and *A. cephalotes* suggest that the copulatory dynamics inferred

from our data might be typical for the entire genus. Via their firm attachment to the queen through the specialized male genitalia, *Atta* males apparently ejaculate sperm into the bursa copulatrix from where it is immediately transferred to the spermatheca. This implies that, although the sperm is ejaculated under pressure, the male still depends on the female for letting the ejaculate pass through the bursa and the spermathecal duct to reach the spermatheca (Fig. 1). The mussel organ is also found in other attine ants (Baer et al., in prep.; Himler et al., submitted), but has never been previously described. The male "saws" may initially have evolved as naturally selected devices to secure attachment between mating partners. However, the same devices could secondarily have been exploited in sexual selection after multiple queen mating evolved. This would have given *Atta* males more control over the mating process, compared to honeybees, where sperm of many males is deposited in the lateral oviducts and gets transferred to the spermatheca with more active control by the queen (Baer, 2005).

Four additional arguments support our contention that sperm is directly transferred to the spermatheca in *Atta* leaf-cutting ants: 1) All queens investigated directly after their mating flight had stored ejaculates in the spermatheca, and only minor remnants of ejaculates were found in other parts of the sexual tract. This was also the case for queens investigated during the mating flight, which were probably still pursuing additional copulations. 2) The morphology of the female sexual tract showed that neither the bursa copulatrix nor the lateral oviducts were large enough to temporarily harbor the ejaculate volume required to completely fill the spermatheca. From our digital pictures we were able to estimate that the volume of an *A. colombica* spermatheca was 28.65 ± 3.30 ($n = 4$) times larger than the volume of the bursa copulatrix. Given that *Atta* queens mate with 2–3 males on average (Fjerdingsstad and Boomsma, 1998; Villesen et al., 1999), an average ejaculate would still be about nine times larger than the volume of the bursa. These are probably even overestimations of the effective bursa volume for hypothetical temporal sperm storage, because the central valve (Fig. 1c) further reduces the available volume in the bursa. Also, the lateral oviducts and the spermathecal duct are not large enough to host a significant fraction of an ejaculate. 3) Sperm is typically seen in bundles within the accessory testes and the same bundles are also visible in the spermatheca shortly after mating, indicating that sperm gets transferred in clumps. This implies that sperm are not individually swimming up the spermathecal duct as in honeybees, where queens store about 1–2 orders of magnitude less sperm than in *A. colombica* and where sperm storage takes 40 h (Baer, 2005). The fact that the *Atta* spermatheca is a noninflated structure in virgin females therefore seems a specific adaptation for rapid

sperm storage. 4) Sperm clumping as found in newly inseminated females of *A. colombica* seems a logical consequence of direct transfer of entire ejaculates to the spermatheca. Since sperm can be seen moving within the spermatheca, it may be that sperm clumping disappears in older queens, but further work will be needed to resolve this issue. The complex muscular tissues found in the bursa copulatrix indicate that this organ may well function as an instantaneous sperm pump immediately after ejaculation.

If this interpretation is correct, we can hypothesize that the evolution of more male control over sperm storage may be characteristic for the unique social evolution that distinguishes *Atta* from most other hymenopteran social insects. As outlined above, direct sperm transfer would have removed the constraint of having to maintain an increasingly larger bursa copulatrix during selection for higher life-time fertility of queens. It would thus have allowed a much larger spermatheca, with many more stored sperm without a significant increase in body size. The fact that *Atta* leaf-cutting ants were able to evolve their enormous colonies may thus ultimately be due to males gaining more control over mating and sperm transfer. However, this hypothesis will need formal testing, for example, by a comparative analysis of bursa and spermatheca sizes across all genera of attine ants.

Direct transfer of ejaculates to sperm storage organs is also known from several nonsocial insects (Simmons, 2001), although males in these species have often also evolved sophisticated traits to remove sperm from the sperm storage sites, of which there is no sign in *Atta* or any other social insect (Boomsma et al., 2005). A similar mechanism of almost direct sperm transfer to the spermatheca has been hypothesized to apply in two species of dwarf honeybees, *Apis florea* and *A. andreniformis*, where males ejaculate into the spermathecal duct from where sperm is transferred to the spermatheca during or very shortly after the copulation (Koeniger et al., 1989; Koeniger and Koeniger, 1991).

Direct transfer of sperm to the spermatheca in the absence of male traits to manipulate previously deposited ejaculates constrains the possibility for further sexual selection. Initial sperm competition is restricted because ejaculates of different males do not compete simultaneously for storage, and sperm competition for egg fertilization after storage will be restricted because ant queens use very few sperm to fertilize an egg (Tschinkel and Porter, 1988). Also, cryptic female choice seems impossible because females do not temporarily store ejaculates prior to long-term storage, a habit that is typical for other (social) insects and which allows females at least some possibility to manipulate the storage process according to their own interest. *Atta* queens also seem to have no possibility to expel stored sperm, because the spermatheca is not surrounded by mus-

cular tissue. The only possibility for females to avoid unwanted matings might thus be precopulatory, by preventing males access to the mussel organ by closing the opening to the sting chamber. This option seems realistic, as the queen's sexual tract is normally blocked by the terminal sternite and tergite, so that it is difficult to open the sting chamber manually unless queens are anesthetized.

An interesting further consequence of direct transfer of ejaculates to the spermatheca is that it imposes unusually strong selection for sperm viability, because less viable sperm cannot be denied access to the spermatheca. Colony size and longevity, and thus queen fitness, are directly dependent on the number of viable sperm stored and on the proportion of this sperm that remains viable throughout the several decades-long life-span of successful queens. We thus predict that *Atta* sperm is of extremely high average quality and has very low variation in viability. It would thus be interesting to compare sperm viability between *Atta* and dwarf honeybees on the one hand, and their respective sister clades (*Acromyrmex* and the other *Apis* bees) on the other. Sperm viability is just one example of a bizarre adaptation that can be predicted from the excessive demands of life-long sperm storage and extreme queen longevity and fecundity in ants. Future studies will undoubtedly reveal more such traits.

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