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The ejaculatory biology of leafcutter ants

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ABSTRACT

The eusocial ants are unique in that females (queens) acquire and store sperm on a single mating flight early in adult life. This event largely determines the size (possibly millions of workers), longevity (possibly decades) and genetic variation of the colonies that queens found, but our understanding of the fundamental biology of ejaculate production, transfer and physiological function remains extremely limited. We studied the ejaculation process in the leafcutter ant *Atta colombica* and found that it starts with the appearance of a clear pre-ejaculatory fluid (PEF) at the tip of the endophallus that is followed by the joint expulsion of the remainder of accessory gland (AG) secretion, sperm, accessory testes (AT) secretion, and a small mating plug. PEF, AG secretion and AT secretion all contribute to sperm survival, but PEF and AG secretion also reduce the survival of sperm from other males. We show that PEF is produced in the AGs and is likely identical to AG secretion because protein-banding patterns of PEF and AG secretion were similar on 1D electrophoresis gels, but differed from the protein-banding pattern of AT secretion. We show that proteins in AG secretion are responsible for the incapacitation of rival sperm and infer that transfer of AG secretion prior to sperm may allow these components to interact with rival sperm, while at the same time providing a supportive biochemical environment for the arrival of own sperm.

1. Introduction

The mating systems of eusocial ants, bees and wasps have a number of characteristics that are rarely, if ever, found in other animals. In almost all species, males complete their life-time production of sperm during the pupal stage and have very short adult lives that often do not allow more than a single day of successful mating (Boomsma et al., 2005; Hölldobler and Bartz, 1985). Queens, on the other hand, have the potential of long reproductive lives during which they can realize astonishing levels of fertility without ever replenishing the sperm that they stored during a single maiden mating event (Baer, 2005; den Boer et al., 2009a; Keller and Genoud, 1997; Pamilo, 1991). To accommodate such high demands for viable sperm, queens possess specialized organs known as spermathecae allowing them to keep sperm alive (Baer et al., 2006, 2009a; den Boer et al., 2010, 2009b; Holman et al., 2011; Kronauer and Boomsma, 2007; Schlüns et al., 2005; Shuker and Simmons, 2014), and sophisticated mechanisms to use just a few sperm to fertilize each egg (den Boer et al., 2009a). These principles of diverging male and female life-spans and life-histories evolved early during eusocial evolution (Boomsma, 2007, 2013; Hughes et al., 2008), and later developed towards spectacular extremes in lineages with very large and long-lived colonies.

Advanced insect societies frequently have colonies living for up to several decades while being headed by the same, often multiply inseminated, queen (Boomsma et al., 2009; Baer, 2011; Jaffé et al., 2012). Polyandry increases genetic variation among the worker offspring, which in turn enhances a colony's collective performance in division of labor or disease resistance (Baer and Schmid-Hempel, 1999; Cole and Wiernasz, 1999; Hughes et al., 2003; Jaffé et al., 2007; Jones et al., 2004; Mattila and Seeley, 2007; Oldroyd and Fewell, 2007; Smith et al., 2008). Such genetic diversity benefits are maximized by sperm mixing in the queen spermatheca and random sperm use, consistent with empirical data (Brodschneider et al., 2012; Franck et al., 1999; Holman et al., 2011; Stürup et al., 2014). This happens in spite of multiple ejaculates being known to damage each other's survival upon insemination, likely because spermathecal secretions eliminate hostile ejaculate components (den Boer et al., 2010). Eusocial mating processes thus appear to be initially driven by conflict between males (sperm competition) and males and queens over sperm storage, but end in peaceful life-long cooperation between stored ejaculates and the queen's egg-laying machinery. The mechanistic details of this transition have remained unknown, but can be hypothesized to be associated

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with structural and functional characteristics of both the male ejaculates during sperm transfer and the queens' reproductive organs.

We examined the ejaculation process in detail, using the leafcutter ant *Atta colombica* as model. Queens of this leafcutter ant are polyandrous and mate with up to seven males (Baer et al., 2006; Evison and Hughes, 2011; Fjerdingstad et al., 1998; Helmkampf et al., 2008). Males contribute different amounts of sperm, and cryptic female choice may further modify the fractions that end up being stored for life (Jaffé et al., 2012; Baer et al., 2003; Fitzpatrick and Baer, 2011). In spite of these sequential processes, stored ejaculates eventually become completely mixed so that harmonious paternity allocation after storage follows a fair raffle (Holman et al., 2011). Continuing promiscuity implies that non-eusocial insects are unlikely to ever reach these forms of reproductive cooperation, but the competitive phases of sperm competition are likely to be comparable. It is reasonable, therefore, to hypothesize that ejaculate competition is mediated by proteins as has been found in non-social insect such as fruit flies (Chapman et al., 2000; Wigby et al., 2009; Fedorka et al., 2011) and that these proteins reside in the seminal fluid (den Boer et al., 2009b; King et al., 2011; Zareie et al., 2013).

We first performed a series of experiments to reconstruct the sequential events that occur during copulation and ejaculation to understand the way in which males assemble an ejaculate and use it to maximize reproductive success. We then studied the separate non-sperm components of the ejaculate to understand their origin and function. Finally, we tested whether the protein or non-protein fractions of seminal fluid are responsible for the phenotypic damage imposed on rival sperm during and just after copulation.

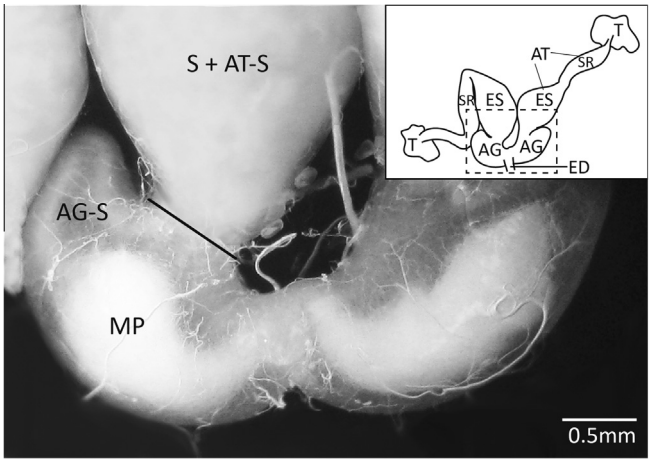

Fig. 1. The reproductive organs of an *Atta colombica* male, with a larger schematic overview of their context (inset). Sexually mature males have degenerated testes (T), accessory testes (AT) where mature sperm is stored prior to ejaculation, paired accessory glands (AG), and an ejaculatory duct (ED) through which the sperm and glandular secretions leave the male sexual tract during ejaculation. The accessory testes are divided into sperm reservoirs (SR) and ejaculatory sections (ES). The photo shows a close up of the paired AGs and part of the paired ATs. A white mating plug (MP) is visible inside the AGs, which are otherwise filled with a clear secretion of the accessory glands (AG-S). The ATs contain sperm (S) and accessory testes secretion (AT-S). The black line marks the transition between the AT and AG sections. AT secretion will only pass this line to mix with AG secretion and to be subsequently ejaculated when muscles surrounding the ATs contract during ejaculation.

2. Material and methods

All ants used for experimental work were excavated from eight mature *A. colombica* colonies in Gamboa, Republic of Panama, in May 2008, 2010 and 2011. We only used sexually mature males, initially tested by dissecting a subsample of males from each colony to confirm that testes were fully degenerated and large quantities of sperm were present in the accessory testes. All dissections were conducted with Inox 5 watchmaker forceps in Hayes saline solution (9 g NaCl, 0.2 g $CaCl_2$, 0.2 g KCl, and 0.1 g $NaHCO_3$ in 1000 ml H_20) (den Boer et al., 2008).

2.1. Obtaining and testing the biological activity of pre-ejaculatory fluid, accessory gland and accessory testes secretions, and proteins in the accessory gland secretion

We used three different approaches to induce ejaculation and reconstruct the course of events during this process. First, we found that decapitating males or separating their gaster from the mesosoma induces intense rhythmic abdominal contractions. These were accompanied by partial extensions of the external sclerotized genitalia, followed by ejaculation in about half of the males. Second, we developed a technique similar to what is used in honeybees for semen collection prior to artificial insemination (Ruttner, 1975). We manually applied gentle pressure from the anterior to the posterior end of the gaster, which resulted in the appearance of semen at the tip of the endophallus. We found this manual collection method of semen to be highly reliable for obtaining ejaculates from different *Atta* species. Third, we provoked ejaculations by dissecting males and removing their sternites followed by gently squeezing the anterior section of the accessory testes (see Fig. 1) with soft forceps.

We found that ejaculation starts with the appearance of a clear drop of pre-ejaculatory fluid (PEF) at the tip of the endophallus (Fig. 2). In order to quantify the effects of PEF on sperm viability, we used 24 pairs of unrelated males, with males in each pair taken from two different colonies. We sampled 1 μl of PEF of each of the 48 males using the manual collection method as described above and added 300 μl Hayes to each PEF sample. Each sample was centrifuged for 5 min at 13,500 rpm and the resulting pellet was discarded to make sure no sperm cells were included in the PEF solution. We then dissected the accessory testes of one of the males per pair and collected three sperm samples of 0.3 μl (Fig. 1) that were then diluted in either: (1) PEF solution of the same male, (2) PEF of the paired and unrelated male, or (3) 300 μl Hayes saline (control). We examined the viability of sperm in each of these samples as described below.

We then separately examined the effect of AG and AT secretion on sperm viability by collecting these secretions from 31 pairs of unrelated males from 5 different colonies. For each pair, we dissected their reproductive tracts and collected one AG and one AT per male and transferred them to Eppendorf tubes both containing 500 μl Hayes saline. The ATs and AGs were carefully ruptured using watchmaker forceps, vortexed for 1 min and centrifuged twice for 4 min at 13,500 rpm to separate tissue and sperm cells from the AG and AT secretions, and the supernatants were transferred into new Eppendorf tubes. We then collected five sperm samples of 0.3 μl each from the other AT of one of the males and mixed them with: (1) the male's own AG secretion, (2) the AG secretion of the unrelated male, (3) the male's own AT secretion, (4) the AT secretion of the unrelated male, and (5) 500 μl Hayes saline as control treatment. Sperm viability was subsequently quantified as described below.

To test whether proteins within the seminal fluid are responsible for the observed phenotypic effects on sperm survival, we collected 25 males from a single colony, dissected their AGs, and pooled all 50 glands in 600 μl Hayes saline. We then ruptured each gland with forceps, vortexed the entire sample for 1 min followed by centrifugation for 4 min at 13,500 rpm. The supernatant

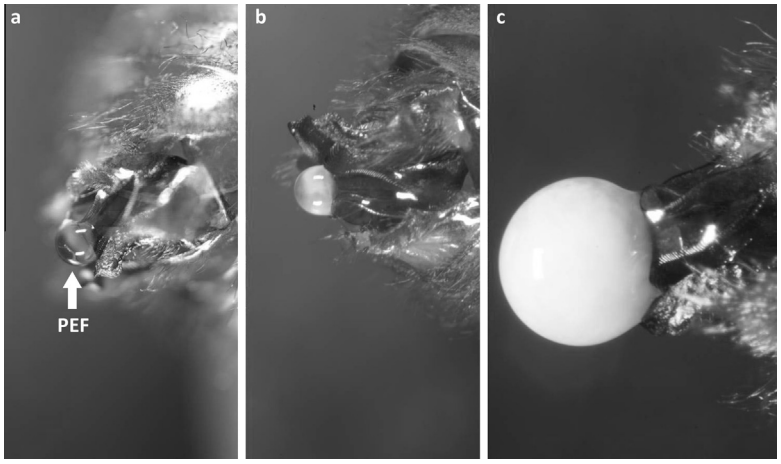

Fig. 2. The ejaculation sequence in *Atta colombica* males in ventrolateral view (a) and lateral view (b and c). Male ejaculation starts with the appearance of a clear liquid (PEF) at the tip of the endophallus (a), followed by the expulsion of increasing amounts of sperm (b and c). The mating plug that appears dorsally of the sperm mass during ejaculation is not visible here.

containing AG secretion was transferred to a Millipore Ultrafree centrifugal filter with a 5 kDa cut-off membrane and centrifuged for 30 min at 13,500 rpm. The filtrate, representing the non-protein fraction, was transferred to a new Eppendorf and Hayes saline was added to obtain a total volume of 600 μl. The protein fraction of AG secretion that had not passed the membrane was collected and re-suspended in 600 μl Hayes. We then aliquoted the two samples, each consisting of 24 μl of protein or non-protein fraction, and added 976 μl Hayes saline to each aliquot resulting in a concentration of approximately one pair of AGs per ml of Hayes.

The AG pairs of an additional 20 males were dissected, collected individually and ruptured in 1 ml Hayes saline. We briefly vortexed each of the 20 samples, centrifuged them for 4 min at 13,500 rpm, and collected the supernatant into a new Eppendorf. Four samples of 1 μl of sperm from one of the ATs were collected from the same males and diluted in: (1) the protein fraction of the AG secretion of different males, (2) the non-protein fraction of the AG secretion of different males, (3) the AG secretion of the same male, and (4) Hayes saline as control treatment. Sperm viability was subsequently measured for each of the four samples as described below.

2.2. Quantifying sperm viability

To quantify sperm viability in the samples described above, we used a Live/Dead sperm viability kit (Molecular Probes) that consists of the membrane-permeate stain SYBR-14 (staining live cells green), and the dead cell stain propidium iodide (staining membrane-compromised cells red). For each measurement, 3 μl of sample was incubated for 5 min with a 3 μl SYBR 14 working solution (2 μl SYBR 14 stock in 98 μl Hayes saline) at room temperature in the dark. We then added 1 μl of propidium iodide and incubated the sample for 4 min before counting the number of green (live) and red (dead) sperm, as well as sperm stained both colors (dying cells) among at least 300 sperm cells using a fluorescence microscope (Olympus CX41, EXFO X-Cite 120, filter cube CX-DMB-2, 400–800× magnification). The number of sperm stained both colors was very low (mean ± s.e.m.; 0.23% ± 0.04%) so we excluded these from further analyses. All sperm viability counts were done blind to sample identity.

When analyzing and interpreting our sperm viability data we followed the recommendation of Holman (2009) to focus on relative differences between treatments and controls within the same experiment, rather than on absolute viability differences within and between experiments. This resolves possible complications emanating from some of our experiments having been done in different years and with males that were possibly of different quality. We always used Hayes saline as a control treatment because it provides a physiologically suitable environment, but without providing any nutritious or energetic support that could enhance sperm cell survival. Any sperm survival values above or below control values could thus be interpreted as reduced or increased mortality due to supporting or damaging compounds in seminal fluid.

2.3. Protein abundance on SDS PAGE gels

To compare the protein compositions of PEF, AT and AG secretion, we visualized protein abundance using 1-D gel electrophoresis. We collected PEF from 30 males using the manual collection method as described above and sampled the first 1 μl of clear liquid appearing at the tip of the endophallus with a pipette. The PEF of all 30 males was pooled in 100 μl Hayes. To collect AT and AG secretions, we sedated males with CO_2 to prevent ejaculation. We then dissected 40 AGs and 40 ATs from 20 males and pooled them in 400 μl Hayes saline each. The ATs and AGs were carefully ruptured using watchmaker forceps. Samples of AT, AG and PEF were then vortexed for 1 min and centrifuged for 10 min at 13,500 rpm after which we collected the three supernatants and transferred them to a new Eppendorf. Each sample was centrifuged a second time for 10 min at 13,500 rpm and the supernatant was collected and stored at −20 °C awaiting further experiments.

For protein precipitation, we added ice cold acetone to each sample and stored it at −20 °C for 24 h. Samples were then centrifuged for 15 min at 13,500 rpm to pellet the proteins, and the supernatant was discarded. To run samples on SDS gels, we re-suspended the proteins in 20 μl of distilled water containing 5 mM Tris (2-carboxyethyl) phosphine (TCEP) and 8 M urea. Estimates of the total quantity of protein in each sample were made using a spectrophotometer (Nanodrop ND-1000). Samples were loaded onto Biorad Criterion precast gels (10–20% Tris HCl, 1 mm, 18 comb) using 15 μg of protein per sample. Gels were run at 180 V until the dye front reached the end of the gel. The gels were then fixed for 12 h in 40% ethanol and 10% acetic acid, and stained afterwards with colloidal Coomassie blue (G 250) overnight. We then washed the gels in 0.5% orthophosphoric acid to remove background staining.

2.4. Statistical analyses

Data were analyzed using SAS 9.1 for Windows (SAS Institute Inc., USA) and SPSS 19 for Windows. To analyze the effects of the various glandular fluids on sperm survival, we used Generalized Estimating Equations (GEE) with a binomial error distribution and a logit-link function, with treatment (the solution that sperm was dissolved in) as repeated measure on the same male.

3. Results

Ejaculation in *A. colombica* follows a distinct sequence of events, starting with the appearance of a clear sperm-free droplet of pre-ejaculatory fluid (PEF) at the tip of the endophallus (Fig. 2a), followed by the expulsion of large quantities of sperm, suspended in the remainder of the seminal fluid (Fig. 2b and c), together with a small mating plug that appears dorsally to the sperm mass. Ejaculations that we observed in dissected males gave the same results, but here we could also observe the rhythmic contractions of the sperm reservoir of the ATs (Fig. 1) just prior to ejaculation, forcing sperm from the sperm reservoir into the ejaculatory section with each contraction (Fig. 1). At the same time, sperm present in the ejaculatory section is pushed through the AGs which forces the AG secretion out to appear first as a clear (PEF) droplet, followed by sperm from the ATs and the mating plug, the latter being visible as a denser white mass within the accessory glands prior to ejaculation (Fig. 1). These observations imply that both PEF and the final mating plug originate from the male accessory glands, which allowed us to conduct a series of experiments to quantify the extent to which distinct ejaculate components affect own sperm survival or ejaculates of unrelated males that would naturally co-inseminate *A. colombica* queens during the same mating flight.

When we examined the biological activity of pre-ejaculatory fluid (PEF), we found the same effects as previously reported for the AG secretion by den Boer et al. (2010). PEF had a significantly positive effect on sperm survival compared to a saline control (Fig. 3; $\chi^2 = 19.97$, $df = 1$, $p < 0.001$), whereas PEF significantly reduced survival of sperm from an unrelated male ($\chi^2 = 6.03$, $df = 1$, $p = 0.014$).

In a next step we tested whether these effects are specific to AG secretions/PEF or whether they are also present in the secretions of the accessory testes (AT). We again found that AG secretion has a positive effect on sperm viability compared to a saline control (Fig. 3, $\chi^2 = 27.49$, $df = 1$, $p < 0.001$), and that AG secretion reduces survival of rival male sperm ($\chi^2 = 8.58$, $df = 1$, $p = 0.003$). However, while AT secretion also had a significant positive effect on sperm survival compared to a saline control (Fig. 4, $\chi^2 = 27.53$, $df = 1$, $p < 0.001$), it did not reduce survival of sperm of rival males ($\chi^2 = 2.43$, $df = 1$, $p = 0.119$). Average sperm survival after exposure to own and non-own AT secretion were statistically indistinguishable to survival after exposure to own AG secretion ($\chi^2 = 0.00$, $df = 1$, $p = 0.969$).

We then examined whether proteins are the components in AG secretion that influence sperm survival. We found that sperm survival was significantly lower in samples exposed to the non-protein fraction of non-own AG secretion, and statistically indistinguishable from sperm survival in the Hayes saline controls (Fig. 5, $\chi^2 = 1.12$, $df = 1$, $p = 0.290$). Exposing sperm to the protein fraction of non-own AG secretion had a more positive effect on sperm survival compared to the non-protein fraction ($\chi^2 = 6.77$, $df = 1$, $p = 0.009$), but some proteins must be responsible for damage to rival sperm, as sperm survival was significantly higher when males were exposed to the proteins of their own AG secretions compared to the AG proteins of a different male ($\chi^2 = 14.76$, $df = 1$, $p < 0.001$).

1D gel electrophoresis of PEF, AG secretion, AT secretion revealed that AG secretion and PEF have similar protein banding patterns (Fig. 6), suggesting that PEF is produced by the AGs and part of the AG secretion. AT secretion, on the other hand, had a distinctly different protein profile compared to AG secretion and PEF (Fig. 5).

4. Discussion

4.1. Reconstructing the dynamics of sperm competition in ants

We have previously shown that the secretion produced by the accessory glands (AG) of males has a positive effect on the same male's sperm survival, but that the magnitude of this effect is reduced when AG secretion interacts with sperm of rival males in *A. colombica* (den Boer et al., 2008; den Boer et al., 2010). The present study details these results. We find that the seminal fluid

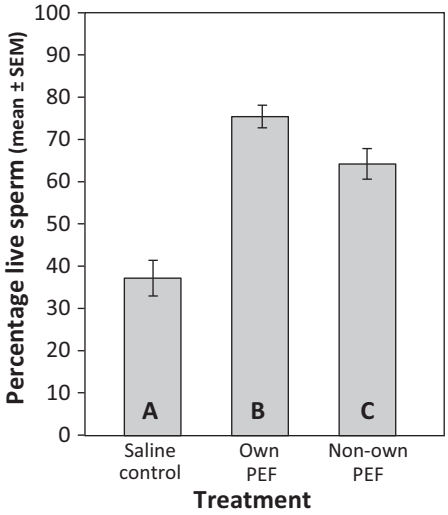

Fig. 3. The survival of sperm cells after exposure to pre-ejaculatory fluid (PEF) of the same or an unrelated male, with mean percentages of live sperm: 37.3% (control), 75.6% (own PEF) and 64.3% (alien PEF). Different capital letters in the bars indicate statistically significant differences (see text for details).

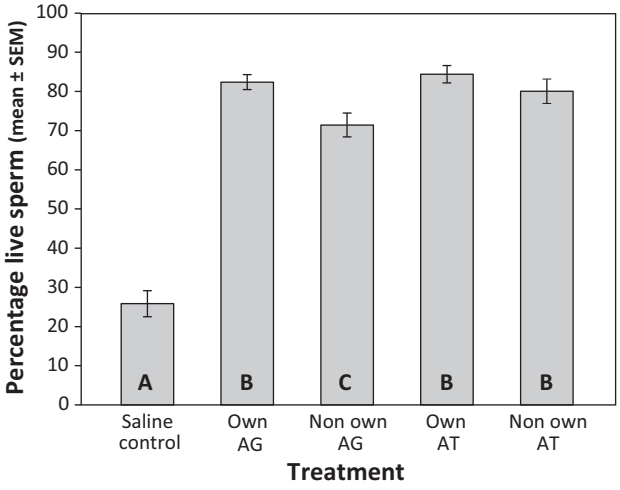

Fig. 4. The survival of sperm cells after exposure to secretion taken from the AGs and the ATs of the same or an unrelated male, with mean percentages of live sperm: 25.8% (control), 82.4% (own AG), 71.4% (alien AG), 84.4% (own AT) and 80.0% (alien AT). Different capital letters in the bars indicate statistically significant differences (see text for details).

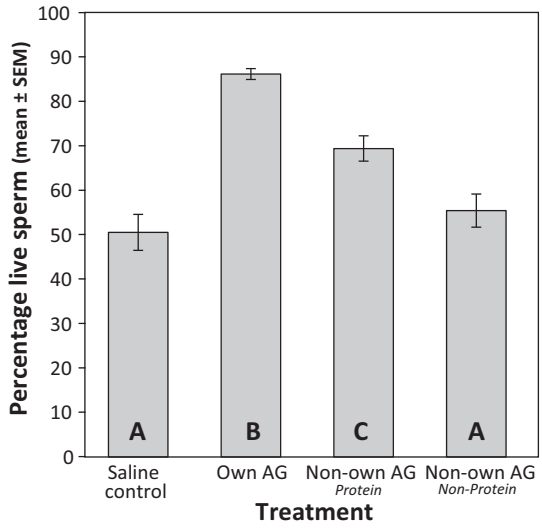

Fig. 5. Effects of the protein and the non-protein fraction of AG secretion on survival of own sperm and sperm from an unrelated male, with mean percentages of live sperm: 50.2% (control), 86.0% (own AG), 69.2% (alien AG, protein fraction) and 55.2% (alien AG, non-protein fraction). Different capital letters in the bars indicate statistically significant differences (see text for details).

(the non-sperm component in ejaculates) is not just made up of AG secretion, but also of secretion produced by the accessory testes (AT, also called seminal vesicles) that is transferred to the queen along with sperm, and a small mating plug. These separate ejaculate components are not transferred as a homogeneous mass, but sequentially and differ in their composition and biological activity.

Prior to ejaculation, sperm is stored in the ATs together with AT secretion. We show that AT secretion has a positive effect on sperm viability. This suggests that its main role is ensuring the survival of sperm in the weeks or days inside the male, before transfer to the queen. As this secretion is already mixed with sperm in the male ATs, it might also offer protection against exposure to compounds

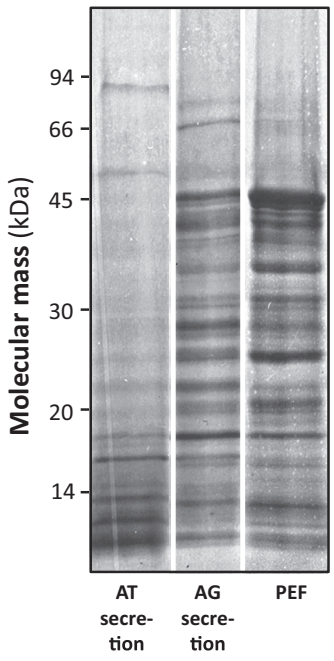

Fig. 6. A 1-D gel visualizing the protein banding patterns (kDa units) after loading 15 μg of spun-down protein of AT secretion, AG secretion and PEF from *Atta colombica* males in each lane. All lanes were cut from the same gel.

in the female reproductive tract during and immediately after sperm transfer. AG secretion, on the other hand, has a different protein composition, is stored separately from sperm cells in the male, and is transferred to the queen just before the sperm, so that AG secretion and sperm only mix during or immediately after ejaculation.

The separation of sperm and AG secretion until ejaculation is likely to have some functional significance. We hypothesize that AG secretion might activate sperm to facilitate transfer to the queen's spermatheca (Poiani, 2006; Smith and Stanfield, 2012). AG secretion could also be transferred first to interact with the female environment before the arrival of sperm, for example to eliminate traces of microbial pathogens that might reside there, as seminal fluid has been shown to contain antimicrobial proteins in a number of insects and mammals (Avila et al., 2011; Baer et al., 2009b; Poiani, 2006). In addition, AG secretion could form a temporary barrier between sperm and ejaculates of rival males already present in the reproductive tract of the queen, to delay potential harmful effects. In spite of this possibility, AG secretion will likely interact at some point with rival male ejaculates already present in the queen reproductive organs and affect their survival as has been shown in non-social insects (Fry and Wilkinson, 2004; Locatello et al., 2013; Price et al., 1999). None of these hypotheses are mutually exclusive and even though we cannot confirm the first three with the present data, we have confirmed the fourth by showing AG secretion's involvement in sperm competition through a reduction in non-own sperm survival (cf den Boer et al., 2010).

4.2. Mating plugs and their putative functions

The mating plug, another product of the AGs, was found earlier to prevent additional inseminations in bumblebees (Baer et al., 2001; Duvoisin et al., 1999) and non-social insects (Bretman et al., 2010; Dottorini et al., 2012; Matsumoto and Suzuki, 1992; Polak et al., 2001), and was suggested to prevent multiple insemination in fire ants (Mikheyev, 2003). However, mating plugs are unlikely to have a similar function in *A. colombica*. The AGs in this species are relatively small compared to the AGs in phylogenetically more basal attine ants that always have single paternity among offspring (Baer and Boomsma, 2004; Villesen et al., 2002). If mating plug size is linearly related to AG size, then the monandrous attine ants are expected to produce relatively large plugs that are successful in preventing re-mating, whereas *A. colombica* males produce relatively small plugs that fail to prevent access of ejaculates of additional males to the queen's reproductive tract. We hypothesize that when multiple mating evolved in the attine ants, selection favored the production of AG components that harm rival sperm to bias paternity after mating, over the production of AG components (mating plugs) that can prevent multiple mating in the first place.

In line with this hypothesis, one would thus expect no investment in harmful components, but more in mating plugs in the AGs of monandrous species, and significant investment in harmful components and less in mating plugs in polyandrous species. This corresponds with our finding that AG secretion is not harmful in a monandrous species, *Trachymyrmex zeteki*, while it is in its polyandrous relatives *A. colombica* and *Acromyrmex echinatior* (den Boer et al., 2010). Nonetheless, the small mating plug in *Atta* could still interfere with a subsequent male's reproductive success, if it could block entrance of rival semen into the spermatheca and/or prevent contact (at least temporarily) between the same male's sperm and harmful AG secretion of a subsequent male to mate with the queen. In *A. colombica*, sperm is directly transferred to the spermatheca without a pre-storage period in the bursa copulatrix or lateral oviducts (Baer and Boomsma, 2006). The lumen of the spermathecal duct is narrow (mean: 0.108 mm, $n = 2$; Den Boer,

unpublished data) and we have never observed mating plugs in the spermatheca of newly mated queens (Den Boer & Baer, personal communication). The sticky mating plug is wider than the spermatheca entrance (mean length 1.44 ± 0.06 mm, mean width 0.796 ± 0.06 mm, $n = 5$; Den Boer, unpublished data) and may therefore block the spermathecal duct and delay the entry of rival ejaculates. At the same time, it could prevent leakage of sperm from the queen's reproductive tract as has been found in other insects (Duvoisin et al., 1999; Polak et al., 1998) and mammals (Jia et al., 2002).

4.3. Other factors that may regulate paternity skew

Leafcutter ant queens maximize their life-time reproductive success by mating with multiple males in spite of the costs (Baer et al., 2006). It would be in their interests to achieve maximal genetic diversity among offspring, which could be realized by storing similar proportions of sperm from all mating partners, a form of cryptic female choice (Jaffé et al., 2012). However this is not what has been found. On the contrary, a skewed representation of sperm from different males has been shown in the spermatheca of *A. colombica* queens (Holman et al., 2011). A first explanation could be that ejaculate sizes are variable and cannot be equalized by the queen in the short time frame between insemination and storage. Indeed, very large differences in sperm complement size have been found in *A. colombica*, both at the colony level and across individual males (Fjerdingstad and Boomsma, 1997; Stürup et al., 2011). Second, ejaculates could achieve different competitive success in being stored, depending on some intrinsic measure of quality (potentially influenced by the queen's reproductive environment). A final factor may be that the first or last males to mate with a queen could have a systematic storage advantage. The clear sequence of AG secretion first, followed by sperm, AT secretion and a small mating plug in *A. colombica*, suggests that the first male to mate with a queen might have an advantage over subsequent males as his sperm will not meet rival AG secretion already present in the spermatheca and his mating plug might delay the arrival of such harmful fluids when the queen mates a second time. In addition, being the first male to mate with a queen might be advantageous as a spermatheca has the capacity to store only a fraction more than what a single ejaculate can provide (Fjerdingstad and Boomsma, 1997; Fjerdingstad and Boomsma, 1998; Baer et al., 2006; Stürup et al., 2011). Fjerdingstad and Boomsma (1998) inferred that a first male to mate with the queen stores on average twice the number of sperm than any subsequent male. However, the number of sperm *A. colombica* males were estimated to transfer to the spermatheca was lower than the average number of sperm in the males' ATs, suggesting that males might also mate multiply.

4.4. Proteins are likely to play key roles in the (in) capacitation of sperm

Our study also documents for the first time that proteins are involved in sperm competition in social insects. The fact that AG proteins have a positive effect on a male's own sperm, but significantly less so on sperm of other males, even if these males are related (den Boer et al., 2010) suggests a mechanism of self/non-self recognition that leads to the incapacitation of rival sperm. Such recognition systems have been found before, for example in seminal fluid – sperm interaction between meiotic drive carrying males and wild-type males in the stalk-eyed fly *Cyrtodiopsis whitei* (Fry and Wilkinson, 2004), in pollen tube formation in plants (e.g. Higashiyama, 2010; Rea and Nasrallah, 2008), and in cooperation between sperm in deer mice, where sperm cells of the same male tend to cluster to a higher extent than mixtures of sperm cells from related and unrelated males (Fisher and Hoekstra, 2010). Self/non-self recognition also seems to play a role in aggregation formation in the budding yeast *Saccharomyces cerevisiae* (Smukalla et al., 2008) and the social amoeba *Dictyostelium discoideum* (Queller et al., 2003). In both species, proteins encoded by a single gene are responsible for the clustering of individuals carrying that same gene. A future step will be to identify the function of separate proteins or groups in their interaction with rival male's semen and the queen's reproductive environment to better understand the molecular mechanism underlying these sexually selected processes.

5. Conclusion

To our knowledge, our study provides the first empirical evidence that proteins in the seminal fluid are responsible for keeping sperm alive during the copulation process. Our results further suggest that accessory gland proteins are instrumental in ejaculate competition and the post-copulatory incapacitation of rival sperm, and that sexual selection has shaped the particular order in which ejaculate components are assembled and transferred to the female. Our findings imply that sperm competition in eusocial insects such as leafcutter ants operates only during a very brief time window between insemination and final sperm storage when queens gain full control over sperm fate and likely have an unambiguous interest in maintaining all sperm. Further proteomic work should now be conducted to identify the biologically active proteins or protein networks involved in the expression and silencing of ejaculate competition in *A. colombica.*

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References

Avila, F.W., Sirot, L.K., LaFlamme, B.A., Rubinstein, C.D., Wolfner, M.F., 2011. Insect seminal fluid proteins: identification and function. Annu. Rev. Entomol. 56 (56), 21–40.
Baer, B., 2005. Sexual selection in *Apis* bees. Apidologie 36, 187–200.
Baer, B., 2011. The copulation biology of ants (Hymenoptera: Formicidae). Myrmecol. News 14, 55–68.
Baer, B., Boomsma, J., 2004. Male reproductive investment and queen mating-frequency in fungus-growing ants. Behav. Ecol. 15, 426–432.
Baer, B., Boomsma, J.J., 2006. Mating biology of the leaf-cutting ants *Atta colombica* and *A. cephalotes*. J. Morphol. 267, 1165–1171.
Baer, B., Schmid-Hempel, P., 1999. Experimental variation in polyandry affects parasite loads and fitness in a bumble-bee. Nature 397, 151–154.
Baer, B., Morgan, E.-D., Schmid-Hempel, P., 2001. A nonspecific fatty acid within the bumblebee mating plug prevents females from remating. Proc. Natl. Acad. Sci. USA 98, 3926–3928.
Baer, B., Schmid-Hempel, P., Hoeg, J.T., Boomsma, J.J., 2003. Sperm length, sperm storage and mating system characteristics in bumblebees. Insectes Soc. 50, 101–108.
Baer, B., Armitage, S.A.O., Boomsma, J.J., 2006. Sperm storage induces an immunity cost in ants. Nature 441, 872–875.
Baer, B., Eubel, H., Taylor, N.L., O'Toole, N., Millar, A.H., 2009a. Insights into female sperm storage from the spermathecal fluid proteome of the honeybee *Apis mellifera*. Genome Biol. 10, R67.
Baer, B., Heazlewood, J., Taylor, N., Eubel, H., Millar, A., 2009b. The seminal fluid proteome of the honeybee *Apis mellifera*. Proteomics 9, 2085–2097.
Boomsma, J.J., 2007. Kin selection versus sexual selection: Why the ends do not meet. Curr. Biol. 17, 673–683.
Boomsma, J.J., 2013. Beyond promiscuity: mate-choice commitments in social breeding. Philos. Trans. Royal Soc. B: Biol. Sci. 368.

Boomsma, J.J., Baer, B., Heinze, J., 2005. The evolution of male traits in social insects. Annu. Rev. Entomol. 50, 395–420.
Boomsma, J.J., Kronauer, D.J.C., Pedersen, J.S., 2009. The evolution of social insect mating systems. In: Gadau, J., Fewell, J. (Eds.), Organization of Insect Societies – From Genome to Sociocomplexity. Harvard University Press, Cambridge, Massachusetts, pp. 3–25.
Bretman, A., Lawniczak, M.K.N., Boone, J., Chapman, T., 2010. A mating plug protein reduces early female remating in *Drosophila melanogaster*. J. Insect Physiol. 56, 107–113.
Brodschneider, R., Arnold, G., Hrassnigg, N., Crailsheim, K., 2012. Does patriline composition change over a honey bee queen's lifetime? Insects 3, 857–869.
Chapman, T., Neubaum, D.M., Wolfner, M.F., Partridge, L., 2000. The role of male accessory gland protein Acp36DE in sperm competition in *Drosophila melanogaster*. Proc. Royal Soc. London B: Biol. Sci. 267, 1097–1105.
Cole, B.J., Wiernasz, D.C., 1999. The selective advantage of low relatedness. Science 285, 891–893.
den Boer, S.P.A., Boomsma, J.J., Baer, B., 2008. Seminal fluid enhances sperm viability in the leafcutter ant Atta colombica. Behav. Ecol. Sociobiol. 62, 1843–1849.
den Boer, S.P.A., Boomsma, J.J., Baer, B., 2009b. Honey bee males and queens use glandular secretions to enhance sperm viability before and after storage. J. Insect Physiol. 55, 538–543.
den Boer, S.P.A., Baer, B., Dreier, S., Aron, S., Nash, D.R., Boomsma, J.J., 2009a. Prudent sperm use by leafcutter ant queens. Proc. Royal Soc. London B: Biol. Sci. 276, 3945–3953.
den Boer, S.P.A., Baer, B., Boomsma, J.J., 2010. Seminal fluid mediates ejaculate competition in social insects. Science 327, 1506–1509.
Dottorini, T., Persampieri, T., Palladino, P., Spaccapelo, R., Crisanti, A., 2012. Silencing of the Hsf gene, the transcriptional regulator of *A. gambiae* male accessory glands, inhibits the formation of the mating plug in mated females and disrupts their monogamous behaviour. Pathogens Global Health 106, 405–412.
Duvoisin, N., Baer, B., Schmid-Hempel, P., 1999. Sperm transfer and male competition in a bumblebee. Anim. Behav. 58, 743–749.
Evison, S., Hughes, W., 2011. Genetic caste polymorphism and the evolution of polyandry in *Atta* leaf-cutting ants. Naturwissenschaften 98, 643–649.
Fedorka, K.M., Winterhalter, W.E., Ware, B., 2011. Perceived sperm competition intensity influences seminal fluid protein production prior to courtship and mating. Evolution 65, 584–590.
Fisher, H.S., Hoekstra, H.E., 2010. Competition drives cooperation among closely related sperm of deer mice. Nature 463, 801–803.
Fitzpatrick, J.L., Baer, B., 2011. Polyandry reduces sperm length variation in social insects. Evolution 65, 3006–3012.
Fjerdingstad, E.J., Boomsma, J.J., 1997. Variation in size and sperm content of sexuals in the leafcutter ant *Atta colombica*. Insectes Soc. 44, 209–218.
Fjerdingstad, E.J., Boomsma, J.J., 1998. Multiple mating increases the sperm stores of Atta colombica leafcutter ant queens. Behav. Ecol. Sociobiol. 42, 257–261.
Fjerdingstad, E.J., Boomsma, J.J., Thorén, P., 1998. Multipe paternity in the leafcutter ant *Atta colombica* – a microsatellite DNA study. Heredity 80, 118–126.
Franck, P., Coussy, H., Le Conte, Y., Solignac, R., Garnery, L., Cornuet, J.M., 1999. Microsatellite analysis of sperm admixture in honeybee. Insect Mol. Biol. 8, 419–421.
Fry, C.L., Wilkinson, G.S., 2004. Sperm survival in female stalk-eyed flies depends on seminal fluid and meiotic drive. Evolution 58, 1622–1626.
Helmkampf, M., Gadau, J., Feldhaar, H., 2008. Population- and sociogenetic structure of the leaf-cutter ant *Atta colombica* (Formicidae, Myrmicinae). Insectes Soc. 55, 434–442.
Higashiyama, T., 2010. Peptide signaling in pollen-pistil interactions. Plant Cell Physiol. 51, 177–189.
Hölldobler, B., Bartz, S.H., 1985. Sociobiology of reproduction in ants. In: Holldobler, B. (Ed.), Experimental Behavioral Ecology G. Fischer Verlag, Stuttgart, pp. 237–257.
Holman, L., 2009. Sperm viability staining in ecology and evolution: potential pitfalls. Behav. Ecol. Sociobiol. 63, 1679–1688.
Holman, L., Sturup, M., Trontti, K., Boomsma, J.J., 2011. Random sperm use and genetic effects on worker caste fate in *Atta colombica* leaf-cutting ants. Mol. Ecol. 20, 5092–5102.
Hughes, W.O.H., Sumner, S., Van Borm, S., Boomsma, J.J., 2003. Worker caste polymorphism has a genetic basis in Acromyrmex leaf-cutting ants. Proc. Natl. Acad. Sci. USA 100, 9394–9397.
Hughes, W.O.H., Oldroyd, B.P., Beekman, M., Ratnieks, F.L.W., 2008. Ancestral monogamy shows kin selection is key to the evolution of eusociality. Science 320, 1213–1216.
Jaffé, R., Kronauer, D.J.C., Kraus, F.B., Boomsma, J.J., Moritz, R.F.A., 2007. Worker caste determination in the army ant *Eciton burchellii*. Biol. Lett. 3, 513–516.
Jaffé, R., Garcia-Gonzalez, F., den Boer, S.P.A., Simmons, L.W., Baer, B., 2012. Patterns of paternity skew among polyandrous social insects: What can they tell us about the potential for sexual selection? Evolution 66, 3778–3788.
Jia, Z.Y., Duan, E.K., Jiang, Z.G., Wang, Z.W., 2002. Copulatory plugs in masked palm civets: prevention of semen leakage, sperm storage, or chastity enhancement? J. Mammal. 83, 1035–1038.
Jones, J.C., Myerscough, M.R., Graham, S., Oldroyd, B.P., 2004. Honey bee nest thermoregulation: diversity promotes stability. Science 305, 402–404.
Keller, L., Genoud, M., 1997. Extraordinary lifespans in ants: a test of evolutionary theories of ageing. Nature 389, 958–960.
King, M., Eubel, H., Millar, A.H., Baer, B., 2011. Proteins within the seminal fluid are crucial to keep sperm viable in the honeybee *Apis mellifera*. J. Insect Physiol. 57, 409–414.
Kronauer, D.M., Boomsma, J.J., 2007. Do army ant queens re-mate later in life? Insectes Soc. 54, 20–28.
Locatello, L., Poli, F., Rasotto, M.B., 2013. Tactic-specific differences in seminal fluid influence sperm performance. Proc. Royal Soc. B: Biol. Sci. 280.
Matsumoto, K., Suzuki, N., 1992. Effectiveness of the mating plug in *Atrophaneura alcinous* (Lepidoptera, Papilionidae). Behav. Ecol. Sociobiol. 30, 157–163.
Mattila, H.R., Seeley, T.D., 2007. Genetic diversity in honey bee colonies enhances productivity and fitness. Science 317, 362–364.
Mikheyev, A.S., 2003. Evidence for mating plugs in the fire ant *Solenopsis invicta*. Insectes Soc. 50, 401–402.
Oldroyd, B.P., Fewell, J.H., 2007. Genetic diversity promotes homeostasis in insect colonies. Trends Ecol. Evol. 22, 408–413.
Pamilo, P., 1991. Life-span of queens in the ant *Formica exsecta*. Insectes Soc. 38, 111–119.
Poiani, A., 2006. Complexity of seminal fluid: a review. Behav. Ecol. Sociobiol. 60, 289–310.
Polak, M., Starmer, W.T., Barker, J.S.F., 1998. A mating plug and male mate choice in *Drosophila hibisci* Bock. Anim. Behav. 56, 919–926.
Polak, M., Wolf, L.L., Starmer, W.T., Barker, J.S.F., 2001. Function of the mating plug in *Drosophila hibisci* Bock. Behav. Ecol. Sociobiol. 49, 196–205.
Price, C.S.C., Dyer, K.A., Coyne, J.A., 1999. Sperm competition between *Drosophila* males involves both displacement and incapacitation. Nature 400, 449–452.
Queller, D.C., Ponte, E., Bozzaro, S., Strassmann, J.E., 2003. Single-gene greenbeard effects in the social amoeba *Dictyostelium discoideum*. Science 299, 105–106.
Rea, A.C., Nasrallah, J.B., 2008. Self-incompatibility systems: barriers to self-fertilization in flowering plants. Int. J. Dev. Biol. 52, 627–636.
Ruttner, F., 1975. Die Instrumentelle Besamung der Bienenkönigin. Apimondia Publishing House, Bucharest.
Schlüns, H., Moritz, R.F.A., Neumann, P., Kryger, P., Koeniger, G., 2005. Multiple nuptial flights, sperm transfer and the evolution of extreme polyandry in honeybee queens. Anim. Behav. 70, 125–131.
Shuker, D.M., Simmons, L.W., 2014. The Evolution of Insect Mating Systems. Oxford University Press, p. 360.
Smith, J.R., Stanfield, G.M., 2012. A seminal fluid protease activates sperm motility in *C. elegans* males. Worm 1, 151–154.
Smith, C.R., Toth, A.L., Suarez, A.V., Robinson, G.E., 2008. Genetic and genomic analyses of the division of labour in insect societies. Nat. Rev. Genet. 9, 735–748.
Smukalla, S., Caldara, M., Pochet, N., Beauvais, A., Guadagnini, S., Yan, C., Vinces, M.D., Jansen, A., Prevost, M.C., Latgé, J.-P., Fink, G.R., Foster, K.R., Verstrepen, K.J., 2008. FLO1 is a variable green beard gene that drives biofilm-like cooperation in budding yeast. Cell 135, 726–737.
Stürup, M., den Boer, S.P.A., Nash, D.R., Boomsma, J.J., Baer, B., 2011. Variation in male body size and reproductive allocation in the leafcutter ant *Atta colombica*: estimating variance components and possible trade-offs. Insectes Soc. 58, 47–55.
Stürup, M., Nash, D.R., Hughes, W.O.H., Boomsma, J.J., 2014. Sperm mixing in the polyandrous leaf-cutting ant *Acromyrmex echinatior*. Ecol. Evol. 4, 3571–3582.
Villesen, P., Murakami, T., Schultz, T.R., Boomsma, J.J., 2002. Identifying the transition between single and multiple mating of queens in fungus-growing ants. Proc. Royal Soc. London B: Biol. Sci. 269, 1541–1548.
Wigby, S., Sirot, L.K., Linklater, J.R., Buehner, N., Calboli, F.C.F., Bretman, A., Wolfner, M.F., Chapman, T., 2009. Seminal fluid protein allocation and male reproductive success. Curr. Biol. 19, 751–757.
Zareie, R., Eubel, H., Millar, A.H., Baer, B., 2013. Long-term survival of high quality sperm: Insights into the sperm proteome of the honeybee *Apis mellifera*. J. Proteome Res. 12, 5180–5188.