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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.

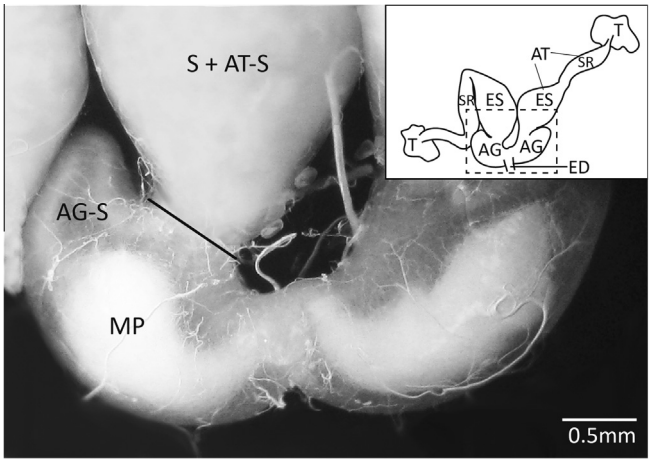

Fig. 1. The reproductive organs of an *Atta colombica* male, with a larger schematic overview of their context (inset). Sexually mature males have degenerated testes (T), accessory testes (AT) where mature sperm is stored prior to ejaculation, paired accessory glands (AG), and an ejaculatory duct (ED) 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