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Proteins within the seminal fluid are crucial to keep sperm viable in the honeybee *Apis mellifera*

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ABSTRACT

Seminal fluid is a biochemically complex mixture of glandular secretions that is transferred to the females sexual tract as part of the ejaculate. Seminal fluid has received increasing scientific interest in the fields of evolutionary and reproductive biology, as it seems a major determinant of male fertility/infertility and reproductive success. Here we used the honeybee *Apis mellifera*, where seminal fluid can be collected as part of a male's ejaculate, and performed a series of experiments to investigate the effects of seminal fluid and its components on sperm viability. We show that honeybee seminal fluid is highly potent in keeping sperm alive and this positive effect is present over a 24 h time span, comparable to the timing of the sperm storage process in the queen. We furthermore show that the presence of proteins within the seminal fluid and their structural integrity are crucial for this effect. Finally, we activated sperm using fructose and provide evidence that the positive effect of seminal fluid proteins on sperm survival cannot be replicated using generic protein substitutes. Our data provide experimental insights into the complex molecular interplay between sperm and seminal fluid defining male fertility and reproductive success.

1. Introduction

Ejaculates consist of sperm as well as glandular secretions referred to as seminal fluid or seminal plasma (Poiani, 2006; Simmons and Siva-Jothy, 1998; Simmons, 2001). The biological function as well as the molecular make-up of seminal fluid has received increasing scientific interest over recent years as several key studies indicated that seminal fluid is important for male reproductive success (Poiani, 2006). Seminal fluid is known to influence a number of different fitness-relevant processes such as enabling the sperm to become fertile (Gillott, 1996) and supporting sperm storage and egg fertilisation (Bertram et al., 1996; Chapman, 2001; Neubaum and Wolfner, 1999; Wolfner, 1997, 2002). Furthermore, components within the seminal fluid are also agents of sexual conflict and manipulate female reproductive physiology and behavior (Baer et al., 2001; Chapman and Davies, 2004; Gillott, 2003; Poiani, 2006; Ram and Wolfner, 2007; Sauter et al., 2001; Simmons, 2001) or sperm competition (den Boer et al., 2010; Wigby et al., 2009).

In insects, the male accessory glands are generally assumed to be major contributors towards seminal fluid and their molecular make up and physiology is intensively investigated. In the best-studied species, *Drosophila melanogaster*, accessory gland secretions are needed to keep sperm alive (Holman, 2009) and more than 100 accessory gland proteins (Acp) have so far been characterized (Ram and Wolfner, 2007). The biological activity is known for some of these proteins (Chapman, 2001) such as Acp62F that has been suggested to inflict a cost of mating for the female (Wolfner, 2002), Acp36DE which is required for successful sperm storage (Bertram et al., 1996) and the hormone Acp26A which stimulates egg laying (Herndon and Wolfner, 1995). The best-studied Acp protein is the sex peptide Acp70A, which inflicts a cost of mating to the female because it affects their post-mating behavior (Kubli, 2003) by inducing male rejection behavior and increasing egg-laying behavior (Chapman, 2001; Chen et al., 1988; Yapici et al., 2008).

Social hymenopteran insects (the social bees, ants and wasps) offer interesting opportunities for the study of seminal fluid and its importance for male reproductive success. Social insect males are under strong selection to produce high-quality ejaculates (Baer, 2005, 2011; Boomsma et al., 2005) because queens receive and store a lifetime supply of sperm early in life, which they never replenish after they start laying eggs (Baer et al., 2006). The sperm storage process is often achieved by a two-level process, where ejaculates are first received and temporarily stored within the

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female sexual tract, before being transferred to the long term storage organ, the spermatheca (Baer, 2003, 2005, 2011; Baer and Schmid Hempel, 2000). This process can take a substantial time, from hours (Duvoisin et al., 1999) to days (Baer, 2005; Ruttner and Koeniger, 1971; Woyke, 1983) and sperm supporting seminal fluid is expected to be crucial for male insemination success during this time. Recent experimental work supported this idea as male accessory gland secretions, assumed to contribute towards seminal fluid (Baer et al., 2009b), maintained sperm viable in leaf cutting ants (den Boer et al., 2008) and honeybees (den Boer et al., 2009), similar to findings in *D. melanogaster* (Holman, 2009). However, all these earlier studies used dissecting techniques and manipulated and macerated male accessory gland tissue mechanically, resulting in unknown levels of molecular contaminations within these samples used for sperm viability measurements. Furthermore, the degree of functional overlap between male accessory gland secretions and seminal fluid has never been quantified in great detail.

Consequently, we used the honeybee *Apis mellifera* and present experimental work that directly uses seminal fluid rather than accessory gland secretions to quantify its effect on sperm survival. Honeybees offer several advantages to perform such work because ejaculates can be sampled directly from ejaculating males (Mackensen and Roberts, 1947; Ruttner, 1976) and seminal fluid can be efficiently separated from sperm using a previously developed centrifugation protocol (Baer et al., 2009b). Recent molecular studies in honeybees reported a number of proteins within the seminal fluid with putative roles in sperm survival (Baer et al., 2009a, 2009b) suggesting that proteins within seminal fluid are important contributors for sperm survival. We therefore specifically tested whether seminal fluid proteins affect sperm viability. We also quantified the effect of seminal fluid on sperm survival over a time frame comparable to that needed for sperm to get stored within the spermatheca. We find that seminal fluid has a consistently positive impact on sperm viability and provide empirical evidence that the presence of proteins and their structural integrity are both crucial for this effect.

2. Materials and methods

2.1. Sample collection

All samples used for experiments originated from mature drones of *A. mellifera* bred at the University of Western Australia between December 2008 and October 2009. Ejaculates were sampled according to a standardized protocol used for artificial inseminations (Baer and Schmid Hempel, 2000; Ruttner, 1976). In short, drones were killed in chloroform to initiate ejaculation. When pressure is manually applied to the drone's abdomen the eversion of the male's endophallus is completed and semen appearing at the tip of the endophallus can be collected using a glass capillary. To collect seminal fluid, a total of five ejaculate samples consisting of 70 μl of pooled ejaculates from approximately 200 drones each were collected. To separate sperm from its surrounding seminal fluid, semen samples were centrifuged at 16,000 × *g* for 20 min at 4 °C and the supernatant was collected and centrifuged a second time at 16,000 × *g* for 20 min at 4 °C to remove any remaining sperm. After collecting the supernatant, protein concentrations were quantified for each seminal fluid sample using a Bradford assay (Bradford, 1976) using a UV mini-1240 spectrophotometer (Shimadzu). All seminal fluid samples were stored at −80 °C prior to any use for experiments.

2.2. Seminal fluid fractionation

To separate seminal fluid into a protein and a non-protein fraction, 60 μl of seminal fluid was used for filtration through a Biomex-5 K column (Millipore) at 7000 × *g* for 10 min at 4 °C. The eluant comprising the non-protein fraction (nPF) of the seminal fluid was collected and stored at −80 °C. The proteins retained in the column (PF) were washed three times by adding 200 μl of Hayes solution (9.0 g/l NaCl, 0.2 g/l $CaCl_2$, 0.2 g/l KCl, 0.1 g/l $NaHCO_3$, pH 8.7) followed by centrifugation at 7000 × *g* for 15 min. The concentration of proteins after the final wash was adjusted to the original seminal fluid protein concentration of 2.7 μg/μl by adding Hayes solution. To visually confirm the presence or absence of proteins in the fractionated seminal fluid samples and to ensure processing did not lead to protein degradation, we ran 2.5 μl of seminal fluid and the fractionated samples on a precast 1-D polyacrylamide gel (Biorad). The gel was run at 20 mA for 3 h, then transferred to a fixing solution (40% methanol and 10% acetic acid) for 1 h before being stained using colloidal Coomassie blue (G 250).

2.3. Sperm viability measurements

Sperm viability was estimated using a previously developed protocol (den Boer et al., 2010, 2008, 2009). In short, sperm samples were collected from drones anaesthetized with CO_2. The seminal vesicles were carefully dissected in Hayes and punctured with watchmaker forceps (Inox 5). A sample of 2 μl of out-flowing sperm was collected with a pipette and diluted in 250 μl of Hayes solution. Sperm viability was assessed using a fluorescent-based nucleic acid staining kit (Invitrogen, L-7011). We first incubated 5 μl of diluted sperm with 5 μl of SYBR-14 on a glass microscope slide in the dark at 25 °C for 10 min, followed by 7 min incubation with propidium iodide. To quantify sperm viability we used a Leica DM 1000 fluorescence microscope at 40× magnification and counted the number of live and dead cells for a minimum of 300 sperm per slide. Sperm viability was calculated as a percentage of live sperm divided by the total number of sperm counted.

2.4. The effect of seminal fluid and seminal fluid proteins on sperm viability

We first tested whether freezing affected seminal fluid function by presenting the sperm of 15 males to either frozen (−80° C) or non-frozen seminal fluid sampled shortly before the experiment. To quantify the effect of seminal fluid on sperm viability we incubated sperm from 40 drones each at different seminal fluid dilutions (1:10, 1:20, 1:100 and 1:500 of seminal fluid). Sperm from additional 40 drones was used separately to test for the effect of the protein and the non-protein fraction of seminal fluid on sperm viability. Prior to sperm viability counts we mixed 2.5 μl of diluted sperm with 2.5 μl of the seminal fluid sample and incubated it for 15 min in the dark at 25 °C before adding the fluorescence dyes as outlined above.

2.5. Sperm survival in seminal fluid over time

To test for the temporal dynamics of seminal fluid on sperm viability we used sperm from 15 drones and measured sperm survival over 30 h, a time span comparable to the sperm storage process inside the queen's sexual tract after mating (Baer, 2005). To do this we split a seminal fluid sample into two sub-samples of 15 μl each and boiled one sample at 100 °C for 3 min to denature the proteins to reduce or eliminate their biological activity. Both samples were then diluted to a final concentration of 0.027 μg/μl protein and used to test for their effect on sperm viability. To do this, 15 males were used and 2 μl of sperm from their accessory testes was diluted in 250 μl of Hayes containing streptomycin (2.5 g/l) and penicillin (0.625 g/l) to prevent microbial growth. We then mixed 2.5 μl of diluted sperm with 2.5 μl of either seminal fluid or boiled seminal fluid. Sperm viability was estimated using