Journal of Insect Physiology 55 (2009) 538–543

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Honey bee males and queens use glandular secretions to enhance sperm viability before and after storage

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ARTICLE INFO

Article history:
Received 11 November 2008
Received in revised form 13 January 2009
Accepted 23 January 2009

Keywords:
Apis mellifera
Ejaculate
Seminal fluid
Accessory glands
Spermatheca
Social insects

ABSTRACT

Internal fertilization requires live sperm to be transferred from male to female before egg fertilization. Both males and females assist the insemination process by providing sperm with glandular secretions, which have been inferred to contain subsets of proteins that maintain sperm viability. Here we show that in the honeybee (*Apis mellifera*) secretions of the male accessory glands, the major contributors towards seminal fluid, enhance sperm survival. We further demonstrate that the protein fraction of the male accessory gland secretion is indeed important for achieving the maximal effect on sperm survival. After sperm storage, the queens also provide sperm with secretions from spermathecal glands and we show that these secretions have a comparable positive effect on sperm viability. SDS gels show that the proteomic profiles of accessory gland secretion and spermathecal fluid secretion hardly overlap, which suggests that males and females use different proteins to enhance sperm viability during, respectively, ejaculation and final sperm storage.

1. Introduction

Producing high quality ejaculates that remain viable during insemination is crucial for males to maximize their reproductive success (García-González and Simmons, 2005; Hunter and Birkhead, 2002), but how males actually influence the viability of their ejaculates remains unclear. In many species, males provide sperm with glandular secretions that are usually referred to as seminal fluid or seminal plasma, but details about the molecular composition of seminal fluid fractions are only available for a few insects such as fruit flies (Ravi Ram and Wolfner, 2007) and honeybees (Baer et al., in press; Collins et al., 2006) and several vertebrates including humans (Fung et al., 2004; Pilch and Mann, 2006). These seminal fluid components can affect both sperm cells and female physiology (reviewed by Chapman and Davies, 2004; Gillott, 2003; Poiani, 2006; Ravi Ram and Wolfner, 2007; Simmons, 2001) and at least some seminal fluid proteins have been predicted to enhance sperm viability and sperm survival (Baer et al., in press; Chapman and Davies, 2004).

Females are also known to provide sperm with glandular secretions. In vertebrates, bovine oviduct secretions have been shown to affect sperm motility and viability (Abe et al., 1995; Satoh et al., 1995) and to enable sperm capacitation to increase fertilization success (King et al., 1994). Females of invertebrate species often possess specialized sperm storage organs (sometimes referred to as spermathecae) where sperm is kept between mating and egg fertilization (Eberhard, 1996; Simmons, 2001). These storage organs are often accompanied by glands and their secretions have been hypothesized to benefit the survival of stored sperm (Prokupek et al., 2008), although neither the molecular composition of these secretions nor their biological activity have been studied in great detail (but see Klenk et al., 2004; Koeniger, 1970; Lensky and Schindler, 1967).

Investigating the mechanisms by which males and females affect sperm viability is particularly interesting in the eusocial Hymenoptera (the ants and some of the bees and wasps). Copulations and insemination are restricted to a single brief mating episode early in a queen's life (Boomsma et al., 2005; Boomsma and Ratnieks, 1996). Males die during or shortly after copulating while queens store large amounts of sperm that remain viable over prolonged periods of time, sometimes for several decades (Keller, 1998; Pamilo, 1991). Reproductive success of males and queens is therefore likely to be correlated with both the quantity and the quality of the sperm cells that females are able to acquire and store (Cole, 1983). Male seminal fluid may thus be particularly important for sperm viability during the provisional storage of ejaculates in the female sexual tract prior to final storage. After transfer to the spermatheca, sperm might have to

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0022-1910/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jinsphys.2009.01.012

survive within the spermatheca for years before it will be able to fertilize eggs. It would thus seem obvious that glandular secretions from the queen's spermathecal glands are also important for sperm viability, but no explicit tests have been done to quantify such effects.

In a recent study on *Atta* leafcutter ants we showed that male accessory gland (AG) secretions, that were inferred to contribute most of the seminal fluid, have a positive effect on sperm viability even when sperm is only exposed to minute quantities of these secretions (Den Boer et al., 2008). In the present study we use the honeybee *Apis mellifera* to further examine this effect, by focusing on proteins within the AG secretion of males. We also test the effect of queen spermathecal secretion on sperm viability and examine whether the proteins produced in male and queen secretions are sex-specific. Honeybees have been a long standing model organism in biology, so that many basal aspects of its mating biology have been studied (for example see Koeniger, 1986; Koeniger et al., 1991; Koeniger and Koeniger, 1991). *Apis mellifera* queens mate with 12 males on average (Tarpy et al., 2004) and store up to 4.7 million sperm (Koeniger and Koeniger, 2000). The process of sperm storage can take up to 40 h (Woyke, 1983), where finally only 3–5% of the sperm is transferred to the spermatheca (Baer, 2005).

2. Material and methods

2.1. Sampling of bees

Bees used for the experiments originated from several colonies of *Apis mellifera carnica* that were kept at the University of Western Australia. Mature males and virgin queens became available during the Australian summer between October 2007 and January 2008, a time span that includes the natural reproductive season of local honeybees. Mature males were collected from male producing colonies. Virgin females were obtained by grafting, whereby 4-day-old female larvae where reared into virgin queens using queenless colonies. Virgin queens were used for experiments at an age of 5–8 days after hatching, which is the typical period for nuptial flights (Ruttner, 1975).

2.2. Dissections and sperm viability measurements

Dissections were carried out with Inox 5 (Biology) watchmaker forceps in Hayes solution (9 g NaCl, 0.2 g $CaCl_2$, 0.2 g KCl and 0.1 g $NaHCO_3$ in 1000 ml H_2O, pH 8.7). Hayes solution was originally developed as a semen extender used for artificial insemination in honeybees and is a relatively simple saline solution that represents an environment that is similar to the inorganic fraction of the ejaculate (Schley, 1987). Because it has no added proteins, carbohydrates, fatty acids or amino acids, Hayes is expected to impose some physiological stress on the sperm cells. This makes Hayes saline ideal as a control solution to examine the positive effects of seminal fluid and spermathecal fluid proteins on sperm survival in an osmotically suitable but slightly suboptimal environment (see also Den Boer et al., 2008).

Sperm viability was measured using a Live/Dead™ sperm viability kit (L-7011, Molecular Probes; Collins and Donoghue, 1999; Den Boer et al., 2008). The kit consists of two fluorescent dyes that allow the experimenter to distinguish live (green emission, using SYBR-14 dye) from dead sperm cells (red emission, using propidium iodide). We used a Leica fluorescence microscope (blue excitation filter at $\lambda = 490$ nm) at 400× magnification and counted the number of live (green), dead (red) and dual-stained sperm cells for at least 400 sperm cells for each sample. Dual stained sperm cells represented on average $0.13 \pm 0.03\%$ (mean $\pm$ S.E.M.) of the total sperm population and were therefore excluded from the data.

2.3. The effect of AG secretion on sperm viability

To investigate the effect of male AG secretion on sperm viability we collected 20 mature males. Each male was killed and his two accessory glands were dissected (Fig. 1A) and placed in 1 ml Hayes saline. The glands were carefully ruptured to help the gland content dissolve into the Hayes saline. The sample was then vortexed and centrifuged for 3 min at 3000 × *g* to separate the soluble gland secretion from the remaining gland tissue. We also collected two sperm samples from the same male by puncturing each of his two accessory testes (also referred to as seminal vesicles, Snodgrass, 1956) in a drop of 4 μl Hayes and obtaining 2 μl aliquots of the out flowing sperm in Hayes, using a micropipette. One of these was then dissolved in the 1 ml Hayes solution containing AG secretion as described above, whereas the second sperm sample was dissolved in 1 ml of Hayes only (control). Sperm viability was then estimated using 5 μl of each sample from which >400 sperm cells were counted (see above).

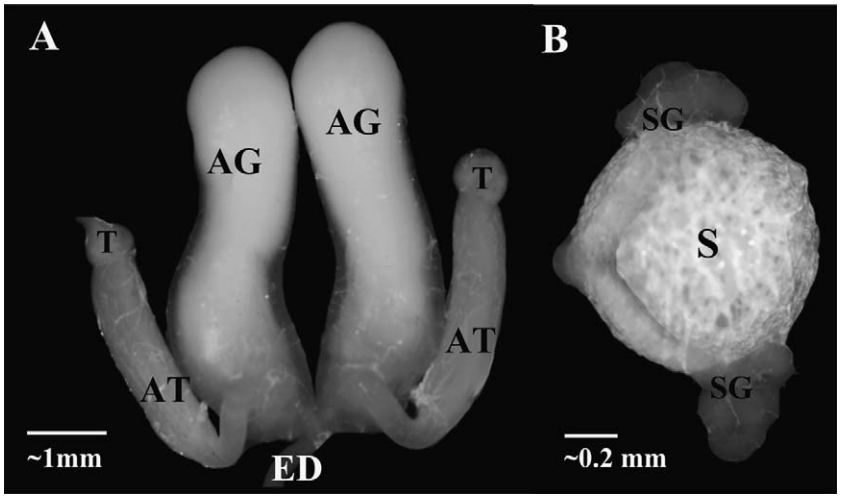

Fig. 1. The sexual organs of an *Apis mellifera* male and queen: (A) The testes (T) degenerate shortly after hatching and sperm is stored in the accessory testes (AT, also known as seminal vesicles). A major part of the seminal fluid is produced in the accessory glands (AG) and mixed with the sperm when an ejaculate is transferred via the ejaculatory duct (ED). The accessory glands also produce a less soluble component, which is typically referred to as mucus and forms a mating plug in honeybees. (B) The spermatheca (S) is covered by a network of trachea and the paired spermathecal glands (SG).