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Female accessory gland fluid promotes sperm survival in yellow dung flies

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Abstract

Female and male reproductive traits co-evolve through pre- and post-copulatory sexual selection and sexual conflict. Although males typically transfer many sperm during copulation, only a small proportion reach the fertilization site because females often actively or passively reduce sperm number in their reproductive tract. Males may transfer accessory substances to protect their ejaculates against female selective processes, which benefits males but can harm females. In turn, females may use accessory gland fluids to control paternity or sperm storage. Female yellow dung flies (*Scathophaga stercoraria*) have paired accessory glands that produce fluids involved in fertilization and egg laying. One proposed function for these fluids is spermicide. Alternatively, female accessory gland fluid may help keep sperm alive to avoid fertilization failure or encourage sperm competition. Using yellow dung flies, we investigated the interaction of female accessory gland fluid with sperm *in vitro*. Significantly more sperm remained alive when exposed to accessory gland fluid compared to buffer only (63% vs. 44%). We conclude that female accessory gland fluid in yellow dung flies can help nourish rather than kill male sperm, although selective nourishment of sperm is as consistent with cryptic female choice as is selective spermicide.

Key Words

Reproduction, sexual conflict, *Scathophaga stercoraria*, sexual selection, sperm competition, spermicide

Introduction

In species with internal fertilization, only a fraction of the vast numbers of sperm transferred by males tend to ever reach the fertilization site (Chang 1951; Hartman 1957; Bedford 1970; Austin 1975; Suarez 1987; Williams et al. 1993; Suarez and Pacey 2006). This sperm loss can result from physiological or biochemical challenges within the female reproductive tract (Birkhead et al. 1993) and, if their tract is large, from additional dilution effects (Immler et al. 2011; Lüpold and Fitzpatrick 2015). Further, females of a diversity of species actively reduce sperm numbers in their reproductive tract by extrusion, dissolution or degradation (Davey 1985; house flies: Degrugillier 1985; bruchid beetles: Eady 1994; spiders: Peretti and Eberhard 2010; *Drosophila* fruit flies: Snook and Hosken 2004; Holman and Snook 2008; Lüpold et al. 2013; Manier et al. 2013; fowl: Pizzari and Birkhead 2000; Dean et al. 2011), implying female influences on sperm storage and paternity that may or may not be adaptive. Therefore, sperm are often short-lived within the female reproductive tract, although there are exceptions such as bees, ants, or bats (Hosken 1997; den Boer et al. 2008; King et al. 2011). In response, males of several insects produce and transfer various accessory substances to protect their ejaculates against female enzymatic attack and digestion (Leopold et al. 1971; Merritt 1989; Duvoisin et al. 1999; Chapman et al. 2001; Lung et al. 2002; Poiani 2006; Holman and Snook 2008; den Boer et al. 2008; King et al. 2011; Avila 2011). These male substances may benefit the males even if to the detriment of the females (i.e., indicating potential sexual conflict; Chapman et al. 2003; Arnqvist and Rowe

2005), for example by decreasing female receptivity to further mating, by accelerating egg laying, storage and use of sperm, or by reducing female life span (Chen 1996; Wolfner 2002; Chapman et al. 1995, 2001).

The female reproductive tract of insects typically includes a pair of ovaries from which the oviducts emanate to further join and form a common oviduct, one to several spermathecae (i.e., sperm storing organs), and paired accessory glands (Wigglesworth 1967; Gillott 1988; Chapman 1998). Davey (1985) documented various functions of female accessory glands: lubrication during copula and oviposition for faster mating and egg laying, production of oviposition pheromones, or protective secretions to coat the eggs. In *Musca domestica*, accessory gland fluid moves along with the spermatozoa to the fertilization chambers and is used to dissolve the cap of the mature egg to allow fertilization (Leopold and Degrugillier 1973; Leopold et al. 1978). Female accessory gland fluid may also facilitate cryptic female choice by creating a selective insemination site (Birkhead et al. 1993; Eberhard 1996; Hellriegel and Ward 1998; Hosken et al. 2001). Several studies have hypothesized or shown that female accessory gland fluids can selectively kill sperm, thus acting as a spermicide (Greef and Parker 2000; Bernasconi et al. 2002; Holman and Snook 2008). A female may benefit from such sperm killing by promoting male competition (Birkhead et al. 1993; Bernasconi and Keller 2001), countering antagonistic male adaptations (Chapman et al. 1995; Rice 1996; Andrés and Arnqvist 2001), or simply by biasing paternity in favour of males of high genetic quality (Birkhead et al. 1993; Greeff and Parker 2000). However, while the number of sperm stored is generally lower than the number transferred (Hosken et al. 2001; Bernasconi et al. 2002), this does not necessarily mean that females actively kill or eject sperm; sperm may simply get lost in the female reproductive tract (Arthur et al. 2008).

The yellow dung fly *Scathophaga stercoraria* L. (Diptera: Scathophagidae) is a cool-climate species that is common around livestock (especially cattle) pastures in cold-temperate regions of the northern hemisphere (Blanckenhorn et al. 2010). In Switzerland, this species abounds up to high altitudes beyond the treeline (Kraushaar et al. 2002). Females lay their eggs into vertebrate dung, which the larvae consume. Consequently, males are usually found in large numbers on and around cow dung pats, waiting for females to mate with (Parker 1970). Due to the pioneering work of Geoff Parker and colleagues, the yellow dung fly has become the classic species for studies of sexual selection, sexual conflict, and sperm competition (Parker 1970, 1978; Simmons et al. 2020). Following copulation with a male, some sperm are stored and partitioned among typically three storage organs (spermathecae) within the female reproductive tract, potentially allowing some level of sperm choice by sorting (Otronen et al. 1997; Ward 2000; Bussière et al. 2010; Demont et al. 2021). Although sperm viability varies in different parts of the female reproductive tract, Bernasconi et al. (2002) found no evidence for female accessory gland fluid affecting sperm viability. One limitation of their approach, however, was that they used previously frozen accessory gland fluid for their *in vitro* experiments. Thus, there is no direct evidence yet that accessory gland products can debilitate sperm in this species (cf. Holman and Snook 2008). To the contrary, it is also conceivable that female accessory gland fluid may actually promote sperm survival (King et al. 2011). Killing sperm by degradation or keeping sperm alive are thus two contrasting functions of female accessory gland products that are both consistent with a female influence on paternity.

Here, we revisited the potential role of accessory gland fluid of female yellow dung fly in sperm viability by using fresh accessory gland fluid to circumvent the possibility of inactivating some important substances by freezing. We predicted two contrasting observations for sperm viability depending on which, if any, of the alternative hypothetical functions for accessory gland fluids is true: compared to a control treatment, we should find more (rather than fewer) live sperm after exposure to accessory gland fluid if accessory glands nourish sperm. In contrast, if the glands promote spermicide, fewer sperm should be alive after exposure to accessory glands than in controls.

Materials and methods

We collected flies from a pasture in Fehraltorf, Switzerland (47°23'N, 8°44'E) and maintained them for multiple generations in the laboratory using standard conditions (Ward 2000; Blanckenhorn et al. 2010). For our experiment we used offspring of the 6th laboratory generation, dissecting a total of 50 females (of which three had to be discarded as dissection was unsuccessful) and 30 males after flies reached sexual maturity (>10 d after adult emergence for females, >4 d for males: Blanckenhorn and Henseler 2005). We performed these dissections in five temporal blocks no more than 12 minutes apart to provide equally fresh sperm, with 10 females and 6 males per block. The fluid of both female accessory glands of each female was extracted by rupturing each gland in a micro-centrifuge tube containing 20 µl buffer (Schneider's *Drosophila* medium; this solution is henceforth referred to as "accessory gland fluid suspension"). We then mixed the extracted accessory gland fluid from all 10 females (i.e., 20 accessory glands) per block so that all sperm samples of the 6 males within a block received the same accessory gland product. To obtain live sperm samples, we dissected individual males and extracted sperm from the proximal end (adjacent to the ejaculatory duct) of one of their testes by piercing the testis and pressing it lightly with a needle until approximately one third of the testis content was released into 100 µl buffer onto a glass slide (Schneider's *Drosophila* medium plus 10% heat-inactivated fetal calf serum: see Bernasconi et al. 2002; this solution is henceforth referred to as "sperm suspension"). All dissections were performed after flies had been anesthetized with CO_2.

To ascertain whether the female accessory gland fluid affects sperm viability, we exposed sperm samples from 30 individual males to both fresh accessory gland fluid mixed with buffer or to buffer alone in a paired design. We incubated 15 µl of the (male) sperm suspension with 30 µl of buffer plus 15 µl of the female accessory gland fluid suspension (or 45 µl of buffer in the control treatment) for a total of 60 µl for 11 ± 2 min at room temperature. Subsequently, we released 30 µl of these mixtures on a glass slide and examined them under a fluorescent microscope. We assessed sperm viability using the LIVE/DEAD Sperm Viability Kit (L-7011, Molecular Probes), which consists of a green membrane-permeant (live) nucleic acid stain (SYBR14, 1 mM in DMSO, diluted 1:50; emission max. 516 nm) and a red stain that penetrates only the damaged membranes of dead sperm (propidium iodide, 2.4 mM in water; emission max. 617 nm). After incubation, we added 5 µl of each stain, vortexed lightly, and incubated the suspension in the dark for 5 min before viewing the sample under the fluorescent microscope. In the rare cases that cells took on both stains, we scored them as dead (Bernasconi et al. 2002). The fluorescent microscope contains three filter sets, allowing the viewing and recording of digital photographs under green light only, red light only, and green plus red light to clearly distinguish dead from live sperm.

We calculated sperm viability as the proportion of live sperm among all sperm counted in the sample (for plotting), based on 20 randomly taken images (frames) per male at 20× magnification within 20 ± 2 min of dissection (corresponding to 9 ± 1 min after adding the stains). Data were analysed with a binomial generalized linear mixed model with logit-link, implemented in in the lme4 package (Bates et al. 2015) of R version 4.04 (R Core Team 2021): the two-vector response variable was the absolute number of sperm that were alive versus dead (summed across the 20 frames taken for each male), and the lone fixed predictor was the experimental treatment. We fitted a random effect for male identity in recognition of the paired nature of the design, and an additional observation level random effect to account for overdispersion in the response. We used parametric bootstrapping (implemented in the pbkrtest package for R: Halekoh and Højsgaard 2014) to assess the significance of treatment.

Results

We counted a mean of 258 ± 53 (SD) sperm per male (sums by treatment: buffer dead: 1819; buffer alive: 1453; accessory gland dead: 1520; accessory gland alive: 2955). The proportion of live sperm was higher when sperm were exposed to accessory gland fluid (mean [± 95%CI]: 0.63 [0.57, 0.68]) than in plain buffer (0.44 [0.38, 0.50]; parametric bootstrap P-value = 0.001; Fig. 1).

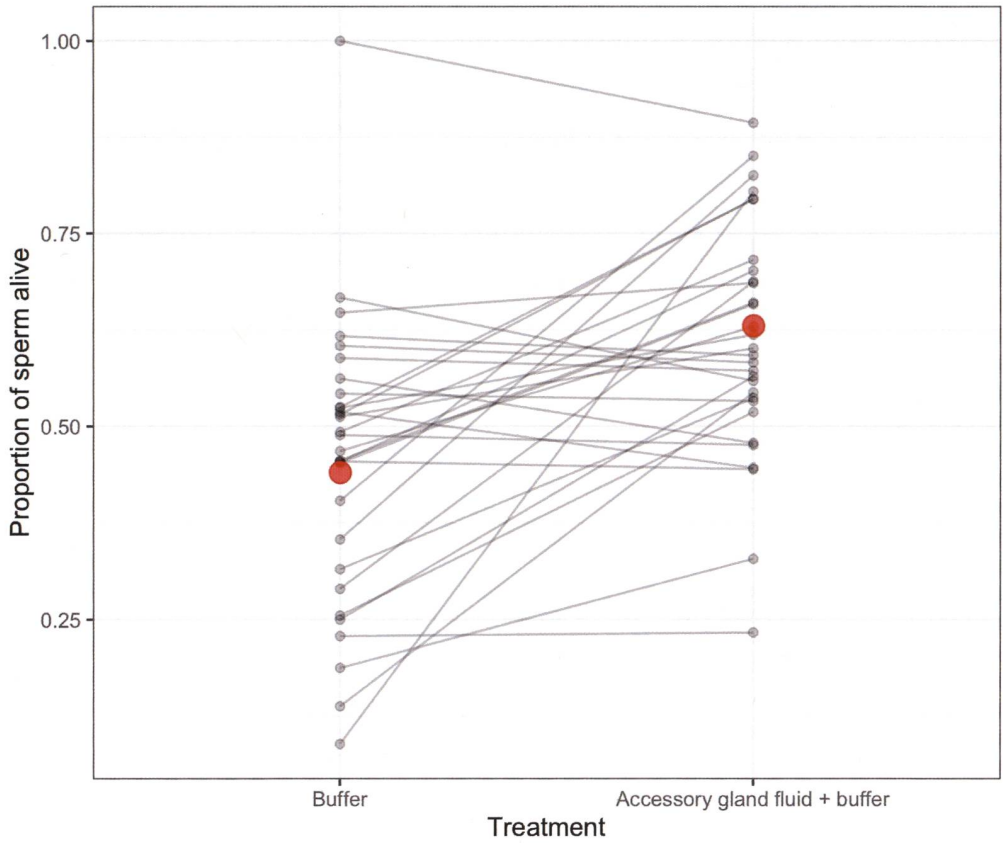

Figure 1. Proportion of sperm from 30 random *Scathophaga stercoraria* males remaining alive after *in vitro* paired treatment with female accessory gland fluid vs. buffer control (red dot = overall mean). Proportions were based on absolute counts of live and dead sperm, which could be distinguished by stain colour, across 20 equal-sized images per male.

Discussion

Bernasconi et al. (2002) had previously shown for yellow dung flies that after mating sperm viability was significantly reduced in spermathecae compared to a male's testes. Nevertheless, *in vitro* exposure of sperm to several parts of the female reproductive tract, including the accessory glands, showed no sperm degradation (Bernasconi et al. 2002). Here, using fresh rather than frozen female accessory gland fluid, we found that this fluid contains substances that apparently increase sperm survival, at least *in vitro*. However, we cannot exclude the possibility that other organs or their secretions may additionally affect sperm viability either inside the spermathecae or in other parts of the female reproductive tract.

Unless there is strict monogamy, which is rare (Birkhead and Møller 1998), the sexes have different reproductive interests, potentially leading to sexual conflict (Chapman et al. 2003). A selective female environment could kill or absorb some incoming sperm before (actively or passively) transferring the remaining sperm to her spermathecae (Hellriegel and Ward 1998). Selective spermicide could be a mechanism to reduce adverse effects of genetic incompatibility (Bishop 1996; Stockley 1999), but could also provide females with a mechanism to bias paternity through cryptic female choice (Birkhead 1998). It is thus possible that accessory gland fluid components with spermicidal functions are female adaptations that arose in the context of sexual conflict, but we found no evidence for this process here.

Sperm are often short-lived within the female reproductive tract. Sperm survival is primarily a function of sperm quality, motility and longevity, and secondarily depends on the female environment such as her accessory gland fluids. Our experiment supports the latter mechanism (without addressing the first). Studies of bees and ants have shown that male accessory fluids can also prolong sperm viability in the female reproductive tract (den Boer et al. 2008; King et al. 2011). The number of sperm transferred by male yellow dung flies during copulation increases with copula duration (Parker and Simmons 1994), and in principle any effect of accessory gland fluid could change with the ratio of sperm-to-fluid. As we diluted the accessory gland fluid in buffer following Bernasconi et al. (2002), we may also have diluted important effects of accessory gland fluid on sperm. However, it seems highly unlikely that the direction of the effect of accessory gland fluid on sperm reverses depending on the concentration of the fluid.

Other studies of insects have found positive effects of female accessory gland fluids on sperm viability and fertilization success. Hosken et al. (2002) showed for *S. stercoraria* that gland extract does not inhibit bacterial growth, suggesting that accessory gland fluid is more likely involved in fertilization functions rather than antimicrobial immunological processes. We stress, however, that selective provisioning of sperm could in principle serve the same function as selective spermicide by creating conditions of sperm storage that favour some males over others. But whether a sperm nourishing function of accessory gland fluid can actively favour certain ejaculates over others remains unclear. Because there is almost always a surfeit of males at the dung, females might not need to keep sperm alive unless they want to impose competition on males they are forced to mate with at the oviposition site. Nevertheless, because keeping sperm alive for weeks inside the reproductive tract may be energetically costly, females may benefit from selective nutrient provisioning of sperm. Females should store only as many sperm as are needed in the short term, and kill or absorb any unnecessary or disfavoured sperm (Birkhead 2000). In yellow dung flies, far fewer sperm are released from the spermathecae during fertilization of individual eggs than was previously thought based on theory (Sbilordo et al. 2009). Along with the abundance of available males willing to mate, this makes sperm limitation unlikely in this species (Simmons et al. 2020).

While our study has clarified one aspect of the function of female accessory glands in yellow dung flies, more work on the physiological and biochemical interactions involved in sperm storage and use, as well as on the reproductive consequences of sperm mortality for male fertilization success and female fitness, is clearly needed to elucidate the multiple facets of sexual conflict and postmating sexual selection in insects.

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Supplementary material 1

Table S1

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Data type: excel table

Explanation note: Sperm counts for 30 males under two conditions, buffer (control) & accessory gland suspension (AG), generated by this study.

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