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Sperm survival in the female reproductive tract in the fly *Scathophaga stercoraria* (L.)

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Abstract

While sperm competition risk favours males transferring many sperm to secure fertilizations, females of a variety of species actively reduce sperm numbers reaching their reproductive tract, e.g. by extrusion or killing. Potential benefits of spermicide to females include nutritional gains, influence over sperm storage and paternity, and the elimination of sperm bearing somatic mutations that would lower zygote fitness.

We investigated changes in sperm viability after in vivo and in vitro exposure to the female tract in the polyandrous fly, *Scathophaga stercoraria*. Sperm viability was significantly lower in the females' spermathecae immediately after mating than in the experimental males' testes. Males also varied significantly in the proportion of live sperm found in storage in vivo. However, the exact mechanism of sperm degradation remains to be clarified. In vitro exposure to extracts of the female reproductive tract, including female accessory glands, failed to significantly lower sperm viability compared to controls. These results are consistent either with postcopulatory sperm mortality in vivo depending entirely on the male (with individual differences in sperm viability, motility or longevity) or with postcopulatory sperm mortality being subtly affected by female effects which were not detected by the in vitro experimental conditions. Importantly, we found no evidence in support of the hypothesis that female accessory glands contribute to sexual conflict via spermicide. Therefore, female muscular control remains to date the only ascertained mechanism of female influence on sperm storage in this species.

Keywords: Female reproductive tract; Sperm storage; Sperm viability; Spermicide; Sexual conflict; Diptera; Female accessory gland; Scatophaga

1. Introduction

In many taxa the number of sperm stored by females is much lower than that transferred by males at mating. For instance, in the beetles *Tribolium castaneum* (Bloch Qazi et al., 1996) and *Callosobruchus maculatus* (Eady, 1994), only 4% and 15%, respectively, of the sperm transferred reach the spermatheca. Females of some species directly extrude sperm after mating (e.g. carrion flies, Otronen and Siva-Jothy, 1991; millipedes, Barnett et al., 1995; feral fowl, Pizzari and Birkhead, 2000). Moreover, the female reproductive tract can be hostile to sperm (Birkhead et al., 1993), reducing the viability and/or fertilization ability of sperm reaching storage, as suggested by evidence of sperm digestion starting immediately after sperm transfer at copulation (flatworms, Michiels and Bakovski, 2000), sperm degradation in the bursa copulatrix (bruchid beetles, Eady, 1994), and that female accessory gland secretions debilitate spermatozoa (house fly, Degrugillier, 1985). Numerical and functional elimination of sperm prior to fertilization counters male interests because males are selected to respond to increased sperm competition risk by maximizing their representation in the fertilization set (Parker, 1990; Hellriegel and Ward, 1998).

Providing a hostile environment for sperm may allow females to (i) promote male competition (Birkhead et al., 1993; Keller and Reeve, 1995; Bernasconi and Keller, 2001), (ii) obtain nutritional benefits (Wickler, 1985; Arnqvist and Nilsson, 2000), (iii) eliminate sperm bear-

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ing somatic mutations (Jones et al., 2000; Siva-Jothy, 2000), (iv) counter antagonistic male adaptations, e.g. to neutralize male-derived substances with detrimental effects on female fitness (Chapman et al., 1995; Rice, 1996; Johnstone and Keller, 2000; Andrès and Arnqvist, 2000), or (v) influence paternity (Birkhead et al., 1993; Birkhead, 1998; Hellriegel and Ward, 1998; Greeff and Parker, 2000).

In the fly *Scathophaga stercoraria*, females have influence over paternity (Ward, 2000a). Theory suggests two mechanisms by means of which females can bias paternity: control of storage rates to multiple spermathecae, and spermicide (Hellriegel and Ward, 1998; Greeff and Parker, 2000). Evidence indicates that sperm storage rates depend on female muscular activity (Simmons et al., 1999), and this results in different proportions of sperm from a female's mates being stored across her multiple spermathecae (Hellriegel and Bernasconi, 2000). In contrast, it is not known whether spermicide occurs, nor are potential mechanisms and their quantitative effects identified. Female tract physiology and products have not been as extensively investigated in Diptera as male-derived products (e.g. *Drosophila* males, Chapman et al., 1995; Rice, 1996). In *S. stercoraria*, female reproductive accessory gland secretions are known to be released during copula (Hosken and Ward, 1999). The glands were found to be larger when females were mated to several males vs. one male after 10 generations (Hosken et al., 2001). Moreover, if sperm are exposed to a hostile environment prior to storage, males may differ with respect to their ability to resist sperm degradation in the female tract, providing a new predictor of variation in paternity success among males. Here we investigate sperm survival after exposure (i) to the female reproductive tract in vivo using virgin males of different age at first mating and (ii) to extracts of different regions of the female tract, including female accessory glands, in vitro.

2. Material and methods

We collected *S. stercoraria* mating pairs in the field (Fehraltdorf, Switzerland, November 1999) and raised their progeny in the laboratory (larvae: 14 °C; adults: 19 °C, light/dark cycle 13/11 hours, Ward and Simmons, 1991). After emergence, virgin flies were kept singly and supplied with water, sugar and *Drosophila* as prey.

2.1. Sperm viability assessment

We assessed sperm viability with the live/dead™ Sperm Viability Kit (L-7011, Molecular Probes), which consists of a membrane-permeant nucleic acid stain (SYBR14; diluted 1:50; emission max. 516 nm) and a dead-cell stain (probidium iodide; emission max. 617 nm). Both solutions stain sperm heads only (Fig. 1). We used Schneider's Medium (GIBCO BRL 21 720-024) with heat-inactivated (30 min/56 °C) 10% fetal calf serum (EUROBIO 01 0056) as buffer. This protocol is an optimization of the protocol of Molecular Probes for our study organism. Samples were analyzed for epifluorescence (Reichert-Jung Polyvar microscope, Hamamatsu C5405, Argus-20). To ensure accuracy, we examined 46 images and recorded them under both light and fluorescence microscopy. Ninety-six percent of the cells seen in light microscopy could be found in the fluorescence image. Overall, 5% (678 out of 13 654 sperm cells) of the cells were doubly-stained (green in centre, red at ends), and were included as dead cells. In a preliminary experiment, we ensured that no dead sperm stain green by heating the testes (10 min/60 °C), which resulted in all cells staining red. We counted live sperm (green, 485/10, BP 540/20) automatically (NIH Image), after calibration to sperm head size in a preliminary experiment (320 images, 4 males). Dead sperm (red, 557.5/27.5) were counted manually, to avoid counting dead tissue of a size similar to sperm heads.

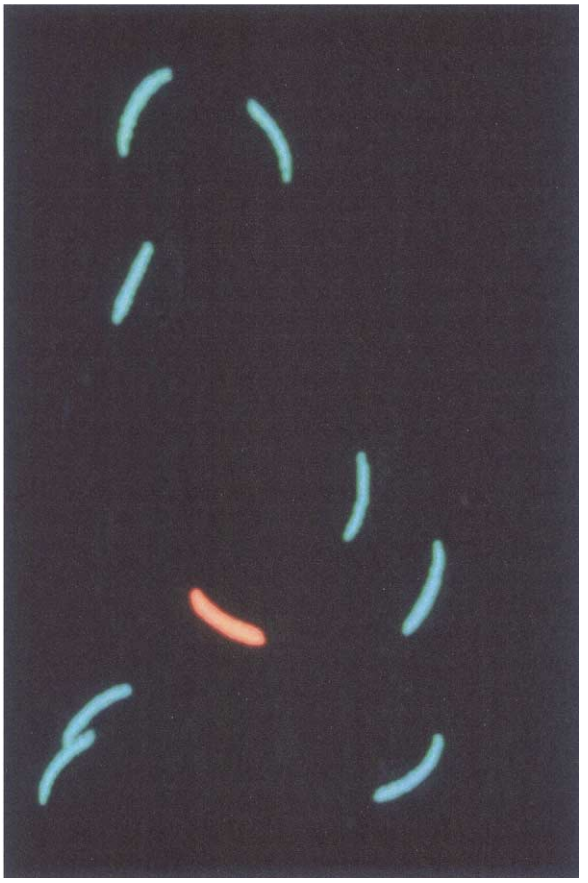

Fig. 1. Live sperm stain green (485/10, BP 540/20), dead sperm red (557.5/27.5; live/dead™ Sperm Viability Kit L-7011, Molecular Probes).

2.2. Sperm viability after in vivo exposure to the female tract

We compared the proportion of live sperm in the spermathecae (i.e. after exposure to the female tract) to the proportion of live sperm in the males' testes for 20 mating pairs. Flies were dissected at the end of copulation (see Hellriegel and Bernasconi, 2000). Because male age could either affect sperm viability in the male or its ability to resist degradation within the female tract, in 10 mating pairs we used young males (18±2 days post-eclosion) and in 10 pairs older males (50±1 days). All males were virgin, thus male age corresponds to age at first mating. The females' spermathecae were placed in 60 μl buffer with 5 μl of each stain, vortexed and transferred to a microslide in 40 μl. Sperm were released by exerting pressure on the cover glass (see control experiment). For the male, we opened each testis separately in buffer on a microslide and released the sperm from the third proximal to the ejaculatory duct. For each testis we added 5 μl of each stain to 60 μl of suspension, vortexed, and transferred 40 μl to a microslide. We counted live/dead sperm on each of 20 (19–21) images for each testis and for the spermathecae. Images were recorded 20±6 min after dissection and 9±4 min after adding stains. Images had 35±23 live and 7±11 dead sperm; 57 out of 1197 (5%) with tissue debris were excluded from analysis. Females were 36±6 days old.

In addition, we carried out a control experiment to ensure that our procedure for preparing sperm from the testis vs. spermathecae did not affect sperm viability. We dissected both testes of each of six males and prepared two separate sperm suspensions which we stained as described for testis (see above). For one of the male's testes we transferred 40 μl of stained suspension to a microslide and examined it directly. For the other testis, we transferred 40 μl of stained suspension to a microslide on which we placed the spermathecae of a virgin female (i.e. containing no sperm) and exerted pressure on the cover glass until the spermathecal walls opened, ensuring comparable pressure as for preparing spermathecal sperm in the in vivo experiment. We examined 320±123 sperm/male (72 images, 6 males).

2.3. Sperm viability after in vitro after exposure to female reproductive tissue

To identify whether, and which part of, the female tract might be responsible for sperm mortality, we exposed samples of sperm (from 24 males, 12 in each of two experimental blocks) to a set of suspensions containing buffer and isolated female reproductive tissues (female accessory glands, spermathecae, bursa copulatrix, bursa copulatrix with female accessory glands) or to a control (male ejaculatory bulb, male flight muscle, female flight muscle, buffer only). All suspensions were kept frozen (−20 °C, 6 days), and thawed and centrifuged (13 000**g**/2 min/4 °C; Haereus Biofuge Fresco) before use. Sperm obtained from virgin males (n=24) were released in 100 μl buffer. We incubated (x±SD: 11±2 min, room temperature) 15 μl sperm suspension with 30 μl buffer and 15 μl tissue suspension. After incubation, we added 5 μl of each stain, vortexed and immediately examined 30 μl of the mixture. We recorded 108 images/male, 5±1 min after staining. Each image had 17±10 live and 4±3 dead sperm in the first and 13±8 live sperm and 3±3 dead sperm in the second block. Results of ANOVA adjusted for covariates (time from testis dissection, p=0.64; duration of incubation, p=0.11) were consistent with the analysis without covariates.

The temporal sequence of all procedures was randomized, sperm counts and incubation sequence were blind. All dissections were under CO_2. Unless specified, data are given as mean±SD. Angularly transformed viabilities were analyzed using GENSTAT 5.3.2 (1995, Lawes Agricultural Trust, Rothamsted Experimental Station) with appropriate F-tests.

3. Results

The proportion of live sperm in the spermathecae immediately after mating and exposure to the female tract was significantly lower than in the male's testes (Table 1, $F_{1,18}$=12.98, p=0.002, Figs. 2 and 3). In the spermathecae, viability was 79.2±10.3% (range 62.2–95.3%), in the testis dissected first it was 88.6±4.7% (80.2–97.1%) and in the testis dissected last 82.7±3.7% (82.7–94.2%). The difference between sperm viability in the spermathecae and the average sperm viability of the two testes was 9.8±11.4%, with the most extreme difference for a given male being 30.1% lower viability in the spermathecae. These results indicate that sperm can die very soon after insemination.

Sperm viability did not differ significantly between a male's testes (Table 1, $F_{1,18}$=0.39, p=0.54), indicating that there was no significant overall difference in the proportion of live sperm between the testis dissected first and second after a single mating (when averaged, the slope of the lines connecting individual values of sperm viability in testis 1 and testis 2 in Fig. 3 is flat). That there was no significant difference between the testis dissected first and second ensures that the procedure to assess sperm viability was rapid enough to avoid artifacts. Sperm survival through storage processes, i.e. the difference between sperm viability in the testes and in the spermathecae, varied widely and significantly among males ($F_{18,1080}$=15.56, p<0.001, Fig. 3). However, this variation was not significantly explained by the age at which males were allowed their first mating (young: 18±2 days post-emergence, n=10; old: 50±1 days, n=10,

Table 1
Accumulated analysis of variance for male age at first mating and sperm source (testes or spermathecae) on angularly-transformed sperm viability in vivo

Source of variation	df		ss	ms	F_{obs}	$p(F>\|F_{obs}\|)$
Male age	1		0.04	0.04	0.05	0.83
Latency to copulation	1		2.12	2.12	2.30	0.15
Copulation duration	1		0.03	0.03	0.03	0.86
Residual among males	16		14.7	0.92		
Source of sperm	2		22.4	22.4	11.17	<0.001
Planned contrasts						
spermathecae vs. testes[a]		1	22.3	22.3	12.98	0.002
testis 1 vs. testis 2[b]		1	0.11	0.11	0.39	0.54
Male age×source of sperm	2		1.99	1.99	1.00	0.38
Individual male×source of sperm	36		36.0	2.0	1.36	0.08
individual male×(spermatheca vs. testes)		18	30.9	1.7	15.56	<0.001
individual male×(testis 1 vs. testis 2)		18	5.1	0.28	1.93	0.01
Residual among images	1080		159.1	0.15		
Total	1139		236.4	0.21		

[a]Effect of exposure to female tract. [b]Variation between a male's testes.

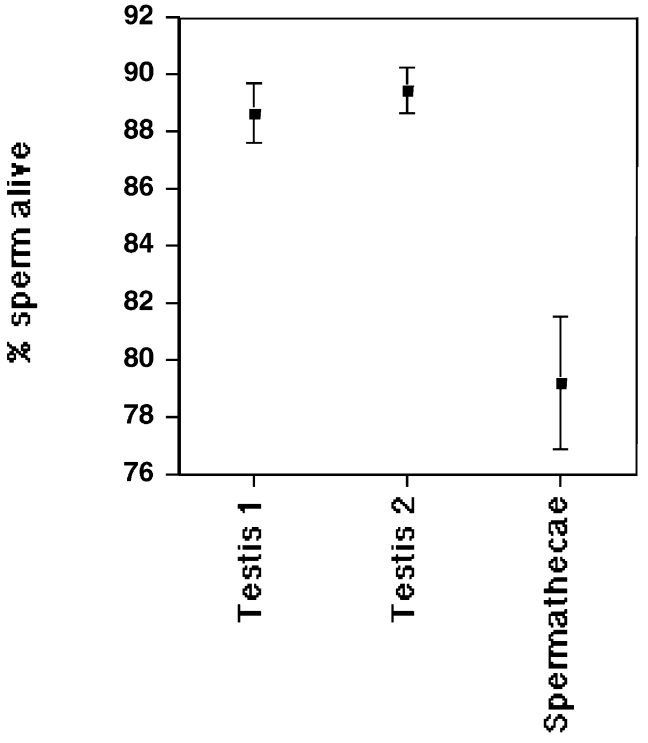

Fig. 2. Viability (mean±SE, n=20 mating pairs) of sperm from the spermathecae of singly-mated females and their mate's testes.

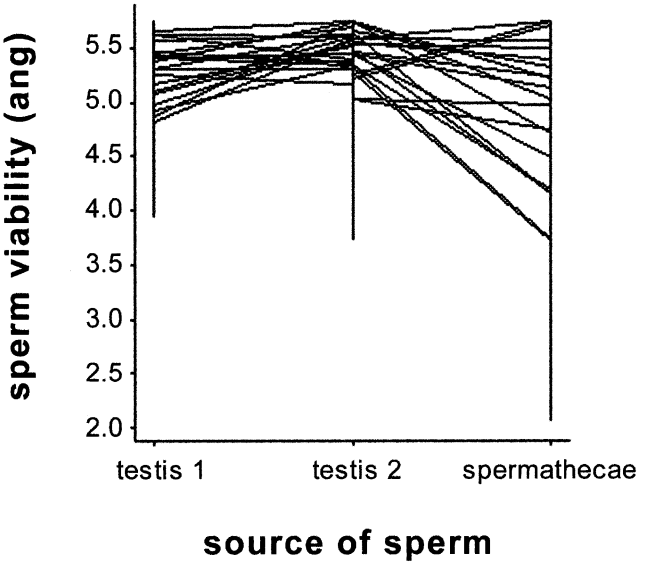

Fig. 3. Effect plot for sperm viability in the testes (1 and 2) of individual males and in the spermathecae of their mate. Lines connect values for each individual male. Sperm viability is angularly transformed [(180/π)×arcsin sqrt(proportion)].

$F_{1,18}$=1.15, p=0.30), nor did male age, latency to (4±4 min) or duration of copulation (36±10 min, n=20) predict viability (all $p \geq 0.15$).

In the control experiments, viability in samples from the testes examined directly after staining (92.7±5.3%) did not differ significantly from viability in samples from the testes on which we applied pressure sufficient to break open a spermatheca (92.0±7.9%; paired t-test: $t=-0.23$, df=5, p=0.83). This indicates that the handling procedures to obtain sperm from the spermathecae vs. from testes did not affect sperm viability differently.

To identify whether parts of the female tract release substances which are toxic to sperm, we exposed sperm in vitro to isolated female reproductive tissues (female accessory glands, spermathecae, bursa copulatrix, and bursa copulatrix with female accessory glands), and to control tissues (male ejaculatory bulb, male flight muscle, female flight muscle, buffer only). Exposure to female reproductive vs. control tissues failed to reveal a spermicidal function of any of these extracts from parts of the female tract ($F_{7,154}$=1.01, p=0.43, 24 males, Tables 2 and 3). None of the linear contrasts comparing each

Table 2
Analysis of variance for angularly-transformed in vitro sperm viability after exposure to female reproductive tissue (female accessory gland, bursa, spermathecae, bursa copulatrix with female accessory glands) vs. controls (male ejaculatory bulb, flight muscles of male and female, buffer only) in two experimental blocks

Source of variation	df	ss	ms	F_{obs}	$p(F>\|F_{obs}\|)$
Block	1	619.2	619.2	0.42	0.52
Residual among males	22	32224.2	1464.7	6.15	
Treatment	7	1683.3	240.5	1.01	0.43
Treatment×block	7	2549.2	364.2	1.53	0.16
Residual among treatments within males	154	36683.0	238.2	1.85	
Residual among images	1529(7)	196734.3	128.7		
Total	1720(7)	270355.5			

Table 3
Sperm viability (mean±SD; %) after in vitro exposure to (a) female reproductive tract extracts and (b) controls in two experimental blocks

(a)	Accessory gland	Bursa copulatrix	Spermathecae	Accessory gland and bursa copulatrix
Block 1	78.7±14.8	83.0±11.5	79.3±14.2	80.5±14.4
Block 2	82.9±15.1	80.9±13.2	82.3±14.7	84.0±13.2
(b)	Male muscle	Buffer only	Ejaculatory bulb	Female muscle
Block 1	83.0±12.1	82.0±13.1	81.4±11.4	82.9±12.6
Block 2	85.7±13.4	82.3±12.6	83.0±14.9	77.1±21.0

female reproductive tissue to the controls was significant (all $p>0.30$). Control tissues did not differ among each other ($p=0.16$, Table 2).

4. Discussion

The proportion of live sperm of a given male was significantly lower in the spermathecae (i.e. after mating and sperm storage) than it was in his testes after copulation. This reduction in sperm viability among stored sperm immediately after a single copulation indicates that sperm degradation already occurs during mating and sperm storage. In extreme cases sperm viability in the spermathecae can be up to 30% less than in the same male's testes, thus potentially putting a male at disadvantage compared to rival males competing for sperm storage and fertilization of the female's ova. By contrast, reduced sperm viability does not necessarily impact female fertility because after one mating females store over 1000 sperm cells (Otronen et al., 1997), which is sufficient to produce at least four fully fertile clutches (Parker, 1970), each of which consists of 55–65 eggs (Blanckenhorn, 2000). The control experiment confirmed that the lower sperm viability observed in vivo in the spermathecae cannot be ascribed to differences in the procedure to prepare sperm for examination. Also, sperm viability in the testes of virgin males (control experiment) was not lower than in the testes of singly-mated males (in vivo experiment). This suggests that low sperm viability in the spermathecae cannot be due to "old" sperm having been transferred at mating. It is also unlikely that the sperm viability difference between testes and spermathecae could arise through preferential storage of dead sperm. Although sperm may be transported up the female tract independently of their motility (Simmons et al., 1999), under female musculature control (Hellriegel and Bernasconi, 2000), it would be counter-adaptive if storage were more efficient for dead sperm. Mechanical damage (sperm are tightly packed in the ducts during transfer and the duct surface is rough, Hosken et al., 1999) or failure in sperm and/or seminal fluid (loss of sperm function, failure in sperm motility, lack of sperm nutrients or necessary cofactors) may also provide an explanation, although selection to avoid such malfunctions clearly should be strong. In sum, the lower sperm viability in the spermathecae than in the testes immediately after mating is an intrinsic property of males (varying in sperm quality, motility, longevity) and/or is mediated by the female tract.

Importantly, we found significant and substantial variation among males in the proportion of live sperm reach-

ing storage. This implies that a male's proportional contribution to the set of stored sperm when females mate multiply (e.g. 2–79% for the second of two males, Hellriegel and Bernasconi, 2000) need not equal his chance of fertilization, because males can also differ in sperm viability and survival to storage. Recent work has focused on understanding sources of variance in male fertilization success (Lewis and Austad, 1990; Arnqvist and Danielsson, 1999) and sexual selection on sperm traits associated with fertilization ability (Keller and Reeve, 1995, Bernasconi and Keller, 2001). Future studies need to establish whether males vary heritably in sperm degradation resistance, and how this correlates with fertilization ability in competitive situations. Interestingly, in the present study variation among males in the proportion of live sperm reaching storage was independent of when males mated for the first time. That is, male age at first mating, and therefore possibly sperm age (Stockley, 1999), did not significantly affect either sperm viability in the male or sperm ability to resist degradation during transfer within the female tract. In *S. stercoraria*, males vary heritably in sperm length, although the causes of this variation are still unidentified (Ward, 2000b). It would thus be interesting to investigate whether variation among males in the sperm ability to resist degradation in the female tract is at least partly explained by variation in sperm morphology.

The mechanism of sperm death remains to be established, as none of the isolated female reproductive tissues (female accessory glands, spermathecae, bursa copulatrix, and bursa copulatrix with female accessory glands) to which we exposed sperm in vitro more strongly affected sperm mortality than control tissues (male ejaculatory bulb, male flight muscle, female flight muscle, buffer only, Table 3). In particular, in vitro the female accessory glands did not have the spermicidal function reported based on preliminary data in a previous study (Hosken et al., 2001). This lack of significance of the in vitro experiment does not allow us to determine whether reduced sperm viability in vivo after storage results from intrinsic male traits or from female influence (whereas a significant spermicidal effect of one or more of the female tract extracts would have strongly supported female influence). In most studies, lack of evidence does not imply lack of effect (Cohen, 1988). However, the statistical power of the in vitro experiment is very high, suggesting that one could conclude that none of the extracts of the female tract we investigated has a spermicidal function. Alternatively, lack of significance can be due to in vitro conditions that did not recreate the relevant physiological conditions, thus masking subtle treatment effects. Indeed, this may have been the case because viability values were generally low in vitro (Table 3).

Recently, female accessory gland were found to be larger in females mated to several rather than one male after 10 generations, suggesting a spermicidal function (Hosken et al., 2001). The present study, however, found no evidence in support of a spermicidal function, despite the high statistical power. Thus, it is possible that these glands, whose secretions are released during mating (Hosken and Ward, 1999), have a different function which is also related to the experimental regime of polyandry/monandry. For instance, lubrication to lower mating costs would be a function that is consistent with the design used by Hosken et al. (2001), which did not separate the two components of polyandry, i.e. mating with genetically different males and mating with more numerous males (see Bernasconi and Keller, 2001, for a design that separates these components). In sum, further work is needed to clarify the function of these glands.

In conclusion, we found significantly lower sperm viability in a female's spermathecae than in her mate's testes. Also, we found significant differences among males in the proportion of viable sperm that their mates store. Sperm mortality during mating and storage can result from intrinsic male properties (such as variation in sperm longevity) and/or from a spermicidal environment in the female tract. However, the mechanism remains to be identified. In vitro exposure to female reproductive accessory glands and other parts of the female tract failed to reveal a female spermicidal effect. Thus, to date, female muscular control of storage rates remains the only ascertained mechanism of differential sperm storage (Hellriegel and Bernasconi, 2000) in this species, confirming at least one of the mechanisms of paternity bias suggested by theory (Hellriegel and Ward, 1998). Indeed, differential storage across multiple spermathecae may be the most flexible mechanism of cryptic female choice at the time of egg-laying in a species where the relevant ecological conditions for optimal choice vary at a microgeographical scale (Ward, 2000a).

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