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Female-mediated differential sperm storage in a fly with complex spermathecae, *Scatophaga stercoraria*

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Multiple spermathecae potentially allow selective sperm use, provided that sperm from rival males are stored differentially, that is, in different proportions across storage compartments. In the yellow dung fly, *Scatophaga stercoraria*, females have three spermathecae arranged as a doublet and singlet. To test whether females store the sperm of rival males actively and differentially, we mated fixed male pairs to three females. After copulation, females were (1) dissected immediately before they could start laying a clutch of eggs, (2) left awake for 30 min but prevented from oviposition, or (3) anaesthetized with carbon dioxide for 30 min to interfere with the muscular control presumably required for sperm transport from the site of insemination to the spermathecae. For each female, we estimated the proportion of the second male's sperm stored in her spermathecae (S_2 value), using sperm length as a male marker. After copulation, the S_2 values in the singlet and doublet spermathecae differed significantly, indicating differential sperm storage during copulation. Postcopulatory treatment affected differential sperm storage significantly. Females dissected immediately had lower S_2 values in the doublet than in the singlet spermatheca, while females left awake showed the reverse pattern for the same two males. This reversal did not occur when females were treated with carbon dioxide. The results indicate differential storage of sperm from different males during copulation and that female muscular activity can affect storage and separation of competing ejaculates beyond copulation.

Females of many species mate with multiple males within a fertile period, leading to competition among rival males' sperm for fertilization of a female's ova (Parker 1970a). Sperm competition may be particularly intense when sperm of several males are stored simultaneously within specialized organs or sites of the female reproductive tract before fertilization (Simmons & Siva-Jothy 1998). Traditionally, sperm competition is seen primarily as an intrasexual conflict. However, female counter-adaptations are expected if intrasexual selection results in male traits negatively affecting female fitness (Chapman et al. 1995; Rice 1996), and/or if males and females have conflicting interests over who is fathering (most of) a female's offspring. A sexual conflict over paternity is especially likely in species where females are forcefully copulated, or where they mate multiply for genetic benefits (Stockley 1997). Where conflict over paternity patterns occurs, it will favour the evolution of female adaptations to influence the fate of her mates' sperm within her reproductive tract (Eberhard 1996). The hostility and complexity of the female reproductive system of many species suggests a function in providing a challenging arena for sperm competition, and in controlling or at least influencing offspring paternity (Eberhard 1985; Birkhead et al. 1993; Hellriegel & Ward 1998). Indeed, there is evidence for significant variation among female genotypes that affects paternity patterns after multiple mating (beetles: Wilson et al. 1997; flies: Clark et al. 1999), for postcopulatory manipulation of ejaculates (beetles: Villavaso 1975; Rodriguez 1994) and for selective sperm use (ascidians: Bishop 1996; lizards: Olsson et al. 1996). The extent of female control over sperm storage and the resulting patterns of paternity are thus likely to depend on both male and female influences (Knowlton & Greenwell 1984; Otronen & Siva-Jothy 1991; Wilson et al. 1997; Clark et al. 1999).

Females may influence paternity by extruding or digesting sperm of different males selectively (Eberhard 1985; Birkhead et al. 1993; Barnett et al. 1995; Haase & Baur 1995), and/or by selectively using suitable adaptations in the physiology, anatomy and function of their sperm

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storage organs (Siva-Jothy & Hooper 1995; Eberhard 1996), sometimes in combination with behavioural adaptations (Siva-Jothy & Hooper 1996). The impact of sperm storage as part of the female reproductive strategy depends on how the sperm reach the storage organ(s), how their viability is maintained, and how they are released at fertilization (Neubaum & Wolfner 1999). In a variety of taxa females have complex sperm storage organs with multiple compartments (Eberhard 1985, 1996), a possible function of which is to enable differential sperm storage, that is, the spatial separation of competing sperm in independent compartments. This separation in turn may allow females to use sperm differentially at fertilization. By choosing sperm from a specific compartment females may favour a particular male during one or more reproductive events. This is especially important if males are not simply good or bad but good depending on, for instance, some environmental factor. Males may counter differential storage by promoting the storage of self-sperm (Bertram et al. 1996; Neubaum & Wolfner 1999). Males may on average aim not only at high, but also equal, representation in all storage compartments, as this potentially decreases variance in their reproductive success (Ward 1993).

In the yellow dung fly, *Scatophaga stercoraria* (Diptera), females are forcefully copulated. Pairs copulate on cow pats (the oviposition substrate) or in the grass, and oviposition starts soon after copulation is terminated (Parker 1970b; Parker et al. 1993). Males ejaculate into the bursa copulatrix, but only those sperm reaching the spermathecae are assumed to be used for fertilization (Simmons et al. 1999). Females have three spermathecae, each with its own duct (see Figure 1 in Ward 1993). Two of the spermathecae are surrounded by a common envelope and partly connected by muscles. They are termed the doublet and the third is the singlet. Paternity patterns show last male advantage, but variation in the proportion of offspring sired by the second mate is high (P_2 range 0–1, e.g. Simmons & Parker 1992). Part of this variance is significantly explained by variation in male traits (Simmons & Parker 1992; Parker & Simmons 1994). Female influences could help to explain the residual observed variance in paternity (Arnqvist & Danielsson 1999), for instance through differential storage and use of competing ejaculates (Ward 1993, 1998; see Simmons & Siva-Jothy 1998 for discussion of the evidence). Sperm motility is low in the Diptera and evidence suggests an active female role in spermathecal filling (Linley & Simmons 1981), possibly through contractions of spermathecal muscles as reported in several insect species (Bloch Qazi et al. 1998; Neubaum & Wolfner 1999). These features are important prerequisites for differential sperm storage.

In this study, we examine the proportion of a second male's stored sperm (S_2 value; Hellriegel & Ward 1998) in the singlet and doublet spermathecae of *S. stercoraria* females to assess (1) whether multiple spermathecae are used for differential sperm storage during copulation, and (2) whether S_2 values can be modified across spermathecae after copulation, and whether they depend on muscular control. Females were doubly mated to fixed male pairs. After copulation had ended, females were subjected to one of three treatments either to allow or to prevent female postcopulatory control over spermathecal muscles. We used sperm length to identify the sperm of individual males. Our experiment combined methods of two earlier studies (sperm identification: Otronen et al. 1997; postcopulatory treatments: Ward 1998). While in singly mated females these treatments mainly affected the total number of sperm stored (Ward 1998), our results demonstrate an additional effect on the distribution of competing sperm across storage compartments in doubly mated females. Specifically, the results indicate that differential storage of competing sperm occurs during copulation and that female muscular activity can affect storage and separation beyond copulation. Sperm storage mechanisms, however, may be under both male and female control.

METHODS

Virgin male and female *S. stercoraria* were collected in 1996 near Fehraltdorf, Switzerland, kept for nine generations in the laboratory and made homozygous for the most common Pgm allele (Ward 1998). Flies were reared and maintained under standard conditions (20°C, 13:11 h light:dark cycle; Ward & Simmons 1991). Adults were kept separate by sex (two individuals per bottle) and supplied with water, sugar and *Drosophila* for 5–6 weeks after emergence to ensure full sexual maturity (Foster 1967). In all matings, we first introduced the female to a bottle containing oviposition substrate (cow dung) to elicit copulation, and subsequently the male. We dissected the flies under carbon dioxide anaesthetic. The flies were killed under the anaesthetic, the thorax and head were briefly squeezed and the abdomen removed for further dissection.

Sperm are monomorphic but their total length varies significantly between males and repeatably within individual males (Ward & Hauschteck-Jungen 1993; Otronen et al. 1997). We therefore used sperm length as an individual male marker to determine the proportion of sperm of each male in the spermathecae. To select males with nonoverlapping 95% confidence intervals in sperm length, we screened interindividual variability by mating 40 males to virgin females. After 20 min, we interrupted copulation and dissected the female. We removed the three spermathecae, transferred them to a drop of insect ringer on a slide and opened them to release sperm. We spread out the sperm, dried the slide, and then washed it to remove salt crystals. We measured the length of 20 sperm per male using the Optimas image analysis software (Optimas 5 1994; 16 × 25 magnification). To assess repeatability of individual sperm length estimates we blindly remeasured 20 (very probably different) sperm on eight slides. Repeatability was confirmed by the significant regression of mean sperm length of the second measurement on the mean of the first measurement (R^2=0.9, $F_{1,7}$=67.2, N=8, P=0.0002). Males were kept for the experiment.

We measured male size as hind tibia length (nearest micrometre, ×16 magnification, measuring table

Completron CX) and body mass (nearest mg, Mettler PM480 DeltaRange). These two measures were significantly correlated (Pearson correlation: r_{37}=0.57, P<0.001; regression equation: mass= − 23.4 (± 16.2) +20.4 (± 4.8) × tibia length; regression coefficient (± SE)), but did not significantly predict mean sperm length of a given male (whole-model test: $F_{2,25}$=1.83, P=0.27; partial regression coefficient for mass ± SE= − 0.05 ± 0.19, t_1= − 0.2, P=0.81, and for tibia length ± SE=0.01 ± 0.01, t_1=1.5, P=0.14). This confirms that male body size and sperm length vary independently of each other (Ward & Hauschteck-Jungen 1993).

Of the 40 males screened, we selected 26 with nonoverlapping confidence intervals of their sperm length to obtain 14 male pairs (two males were used in two different combinations with 2 days for recovery in between). We randomly assigned three females to each male pair (N=42 females in total). Within pairs one randomly selected male was used as the first mate for all three females. The three females were randomly assigned as first, second and third mate of a male pair. Thereafter, we randomly distributed the three postcopulatory treatments among females (see below). Randomization of both the mating sequence of the three females and of their assignment to a treatment is important if male behaviour changes with subsequent matings. Males mated four times within 2–4 days with the last three matings in a row, a mating regime for which sperm depletion is unlikely (Ward & Simmons 1991). Females were allowed an intermating interval of 1 day (19–23 h; as Simmons et al. 1999), during which they had no oviposition substrate and no eggs were laid. Both the first and the second mate were allowed full copulations. We recorded copula duration as observable genitalic contact.

At the end of their second copulation, females were separated from males and (1) dissected immediately, (2) left awake for 30 min and prevented from oviposition, or (3) treated with carbon dioxide for 30 min before dissection. In insects, carbon dioxide anaesthesia does not affect sperm motility (Bloch Qazi et al. 1998) but inhibits muscular activity so that any active muscular-dependent transport of sperm by parts of the female reproductive tract ceases. Muscles surround the bursa copulatrix and spermathecal ducts, and connect the spermathecae to the oviduct (Hosken et al., in press).

Differential sperm storage occurring during copulation (treatment 1) potentially affects paternity when oviposition directly follows copulation. Treatments 2 and 3 allowed us to assess whether muscular control is involved in sperm storage. Sperm storage processes continuing beyond copulation might affect the paternity of either future clutches, or of delayed oviposition. Not all copulations occur directly on cow pats (Parker et al. 1993), suggesting that the delay between mating and oviposition might vary under natural conditions. However, the magnitude of this delay and its variation have not been estimated. We therefore chose 30 min following a previous study (Ward 1998). After treatment, we dissected the three spermathecae as done for screen matings, except that we spread the sperm from the singlet and doublet on separate slides. We measured 84 ± 27 ($\bar{X}$ ± SD) sperm per slide. Measurements were carried out blindly with respect to male pair and treatment.

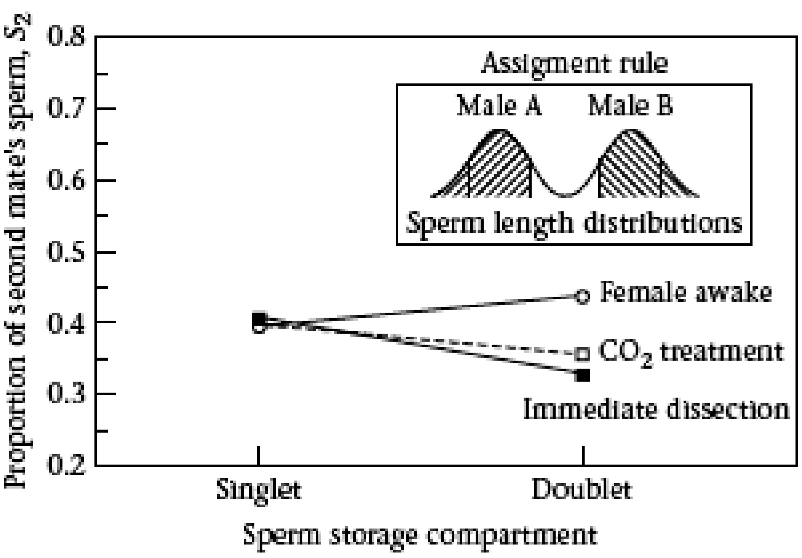

Figure 1. Interaction plot for the proportion of a second male's sperm (S_2) in the singlet and doublet spermathecae of doubly mated females (SEs 0.07–0.09). S_2 values were estimated by assigning measured sperm in the dashed areas to either of two rival males (A, B) using previously determined 95% confidence intervals of individual sperm length.

We estimated S_2 values separately for singlet and doublet spermathecae as the number of measured sperm attributable to the second mate divided by the total number of sperm assigned. Sperm were assigned to either of the rival males (A, B) using one-sided 95% confidence intervals of sperm length (Fig. 1, inset). That is, we included sperm shorter than the lower bound of the left confidence interval (male A), and sperm longer than the upper bound of the right interval (male B).

We analysed the effect of treatment on differential sperm storage in the spermathecae using a doubly repeated measures MANOVA. The three female treatments and the two storage compartments were used as repeated measures of the same male pair (Sokal & Rohlf 1981; SPSS 1990) and S_2 values as the response. S_2 values were angularly transformed before analysis (arcsine ($\sqrt{S_2}$); Sokal & Rohlf 1981). Figure 1 depicts untransformed values. When variances were not homogeneous, as indicated by a significant Mauchly sphericity test, multivariate tests and Wilk's lambda were used (SPSS 1990). Following standard statistical procedures we first examined the significance of the interaction term (read Table 3 in the Results from bottom to top; Sokal & Rohlf 1981). A significant interaction implies that the main factors have nonadditive effects. Significance or nonsignificance of the main effects does not affect the interpretation of a significant interaction term and therefore the main effects have to be considered in this interpretation irrespective of whether they are significant (Sokal & Rohlf 1981). For the analyses we used SPSS 4.0 for the Macintosh (SPSS 1990). All probabilities are two tailed.

RESULTS

Fourteen pairs of males with nonoverlapping sperm length were each mated with three females. The mean

Table 1. Phenotype and sperm length of competing males ($\bar{X}$±SE from 14 double matings)

Mating order	Male body mass (mg)	Tibia length (μm)	Mean sperm length (μm)
First male	47.5±1.1	3391±37	206.7±1.0
Second male	44.6±1.8	3385±45	207.0±1.6
	t_{13}=1.39, P=0.19	t_{13}=0.10, P=0.92	t_{13}=−0.12, P=0.91

Tests are paired *t* tests.

Table 2. Copula duration in min ($\bar{X}$±SD) in relation to postcopulatory treatment of the female

	Awake for 30 min	Dissected immediately	CO_2 treated for 30 min
Copula duration male 1	32.9±11.3	31.6±11.3	28.4±8.9
Copula duration male 2	32.5±10.4	36.4±3.6	32.6±10.1
Ratio of copula durations (male 2:male 1)	1.09±0.43	1.28±0.62	1.28±0.63

difference in sperm length between rival males ± SE was 6.5 ± 0.8 μm (range 1.7–12.8, N=14). With respect to male mating order (male 1 or 2), there was no significant bias in body mass, body size or sperm length, indicating that randomization was successful (Table 1).

Copula Duration

Each male was mated in sequence to three females, either always as first or as second mate. Copula duration with already mated females and with virgin females did not differ significantly (Table 2; repeated measures ANOVA, between-subjects, male 1 or 2: $F_{1,26}$=1.29, P=0.27). Postcopulatory treatment groups were homogeneous with respect to copula duration of rival males (within-subjects: treatment: $F_{2,52}$=0.79, P=0.46; treatment × male 1 or 2: $F_{2,52}$=0.50, P=0.61), as expected from randomization of mating sequence and treatment group. The ratio of copula durations of rival males was not significantly correlated with S_2 values (Spearman rank correlation: all P>0.50).

Estimation of S_2 Values

A mean ± SD of 79 ± 16% (range 50–99%) of the sperm measured in the singlet spermathecae, and 80 ± 17% (range 44–99%) of the sperm measured in the doublet spermatheca could be assigned to either of the rival males; in total, 5630 (79%) of 7096 measured sperm could be assigned to the rival males. This suggests that most of the information content of sperm length variability (screened in singly mated females) could be effectively used to estimate S_2 values in the spermathecae of doubly mated females, although inevitably some sperm cells fall in the large region of nonoverlap between intervals. The resulting S_2 values were extremely variable (range 2–79%) with overall mean of 38.5%.

Differential Sperm Storage and Postcopulatory Treatment

Experimental treatment after copulation significantly affected sperm storage across the spermathecae of a given female, as indicated by a significant interaction of female treatment and storage site on S_2 values (Table 3). The interaction plot (Fig. 1) illustrates how differential sperm storage (S_2 values) in the singlet and doublet spermathecae depended on postcopulatory treatments. In the doublet spermathecae the mean S_2 value of females awake after copulation (0.44 ± 0.09) was higher than in both other treatments (carbon dioxide-treated: 0.36 ± 0.08; immediately dissected: 0.33 ± 0.07). By contrast, in the singlet spermatheca the S_2 values were very similar for all treatments (awake: 0.39 ± 0.09; carbon dioxide-treated: 0.40 ± 0.08; immediately dissected: 0.41 ± 0.08).

Significantly more of the second male's sperm were stored in the singlet than in the doublet spermathecae of females dissected immediately after copulation (Fig. 1; paired t test: t_{13}=2.5, P=0.027; Bonferroni correction accounting for use of these data also in MANOVA, see above, leads to a nominal α=0.025 and borderline significance). This indicates that sperm storage occurs during copulation and that it involves differentially the compartments of the female's complex sperm storage organ. While for immediately dissected females the S_2 value in the doublet was lower than in the singlet, there was no significant difference between compartments for females treated with carbon dioxide (paired t test: t_{13}=1.0, P=0.34) and awake females (paired t test: t_{13}=−1.3, P=0.21). The tendency was if at all in the opposite direction for females left awake. Females treated with carbon dioxide had very similar S_2 values in both storage compartments. Thus, compared with the situation immediately after copulation, the proportion of the second male's sperm stored in the singlet versus the doublet changed in the following 30 min.

Table 3. Postcopulatory influences on the proportion of a second male's sperm in the spermathecae (MANOVA table for angularly transformed S_2 values using three postcopulatory treatments and two storage compartments as repeated measures)

Source of variation	*df*	Mean square	*F*	*P*
Between subjects:				
Covariates*	1	0.98	1.26	0.28
Within subjects:				
Covariates	1	0.01	0.71	0.42
Storage site (singlet, doublet)	1	0.01	1.31	0.27
		Wilk's lambda		
Covariates	4,20	0.53	1.90	0.15
Treatment	2,10	0.96	0.21	0.81
Covariates	4,20	0.79	0.61	0.66
Treatment×store	2,10	0.45	6.17	0.02

*Covariates: sperm length, tibia length and body mass of first and second male to mate.

When the overall last male success at storage was calculated as the mean S_2 value of both storage compartments, there was no significant difference between postcopulatory treatments (paired t test: all $P>0.30$; awake: 0.42 ± 0.9; carbon dioxide treatment: 0.38 ± 0.08; immediate dissection: 0.37 ± 0.08).

DISCUSSION

Our study revealed that the proportion of the second mate's sperm (S_2 value) was significantly higher in the singlet than in the doublet spermatheca immediately after copulation. This indicates that sperm of rival males are stored in different proportions across spermathecal compartments during copulation, possibly because of female muscular control. Uneven storage could influence paternity patterns in the clutch laid directly after copulation, depending on how the three spermathecae are used for fertilization. An uneven distribution of rival sperm is not expected if spermathecal filling is under male control as is traditionally assumed for this species (e.g. Parker et al. 1990). Therefore, our results strongly suggest an additional female effect. The observed difference in S_2 values between storage compartments is consistent with several possible physical mechanisms by which sperm can reach the spermathecae from the site of insemination (the bursa). Our finding of differential storage, however, clearly shows that the female reproductive tract does not work as one unit during this process, providing a possible functional explanation for the evolution of complex, multiple-chamber spermathecae in this species. Although by itself differential storage does not provide evidence for cryptic female choice (Birkhead 1998), it may provide a mechanism for later differential sperm use (Ward 1998).

The significant effect of postcopulatory treatment on S_2 values across spermathecae indicates that female muscular control is involved in sperm storage processes after copulation. While females dissected immediately after copulation had the lowest proportion of the second male's sperm (S_2 value) in their doublet, females left awake for 30 min had the highest. This difference suggests that, at least when immediate oviposition is prevented, the process of sperm storage continues beyond copulation. The pattern of differential storage observed in immediately dissected females disappeared when, for the 30 min after copulation, female muscular activity was either allowed, or prevented by treatment with carbon dioxide. The functional explanation for this change in the male's proportional representation across spermathecae when oviposition after copulation is experimentally prevented for 30 min remains to be clarified, and depends on natural variation in the delay between mating and oviposition. The mechanisms responsible for this change in sperm storage patterns might differ between treatments. This is suggested by the finding that in singly mated females treatment with carbon dioxide results in an increase in the overall number of stored sperm compared with immediately dissected females, while the awake treatment does not (Ward 1998). In fact, that changes occurring after copulation (in awake and carbon dioxide-treated females) affected only the doublet but not the singlet has an interesting parallel in *Drosophila melanogaster*, in which sperm displacement occurs in only one of the three sperm storage compartments (Price et al. 1999).

The differences between treatments cannot be ascribed to differences between males or mating sequence effects. Each male pair was used in all treatment groups (as recommended by Lewis & Austad 1990 and Birkhead 1998), and female treatments were randomized with respect to both mating sequence and treatment group. Moreover, in our experiment males were all large and they were randomized with respect to body size and sperm length between mating roles (Otronen et al. 1997). Randomization was successful in avoiding any bias in these variables. Furthermore, muscular control of parts of the female reproductive tract is in agreement with the finding that female nervous control is required for normal sperm storage in *D. melanogaster* (Arthur et al. 1998), and with evidence that in the Diptera spermathecal filling appears to depend on an active role of the female reproductive system rather than on sperm motility (Linley & Simmons 1981). In *S. stercoraria*, sperm transfer to the bursa increases linearly with copula duration (Simmons et al. 1999). Postcopulatory treatment groups were homogeneous with respect to copula duration of both males. Thus, variation in copula duration between matings is unlikely to explain the effect of postcopulatory treatment. There was also no significant correlation between copula duration and S_2 values.

Our estimates of S_2 values are based on repeatable interindividual variability in sperm length, allowing assignment of roughly 80% of the measured sperm cells to the rival males. We validated this procedure by assessing repeatability of measurements and measuring blindly with respect to male pair and female treatment. The estimated S_2 values are relatively low (grand mean: 0.4) compared to previously reported P_2 values with a mean of 0.8 (Parker 1970a; Ward 1993). However, our results are consistent with the mean S_2 value obtained for the same population in another study (S_2=0.53, as calculated from Figure 2 in Otronen et al. 1997). The S_2 value of a spermatheca and P_2 value are equivalent only if the female uses sperm from this spermatheca for fertilization, and if fertilization occurs according to the males' representation in this spermatheca. There are at least three possible explanations for our low S_2 values. First, sperm length may not be a neutral trait with respect to sperm storage. However, when included as a covariate in the analysis, sperm length did not show significant effects. Moreover, the results on differential sperm storage and postcopulatory influences would not be affected by sperm length because we used the same male pair in each of the treatment groups. Second, sperm may not mix homogeneously in the storage compartments, resulting in a positional effect that favours the last mate at fertilization. Positional effects may be amplified by locally different sperm digestion rates, as found in Orthoptera (Longo et al. 1993). In *D. melanogaster*, however, sperm seem to mix freely in the storage organs (Price et al. 1999). Third, at least in principle, high P_2 values could be caused by sperm from the bursa being used for fertilization, although this is very unlikely (Simmons et al. 1999).

When the S_2 values of singlet and doublet spermathecae were averaged as a measure of the potential overall success of the second male, the resulting mean remained roughly constant across treatments. This suggests that differential sperm storage might allow significant separation between the sperm of rival males without strongly influencing the overall male representation. However, the outcome of male–female conflict and male reproductive success will depend crucially on how sperm from the spermathecae are used for fertilization. This is a central issue that needs to be addressed. The storage compartments may be used simultaneously, at random, or preferentially over a single or successive fertilization events. Preferential use of sperm from the singlet over the doublet and over sperm from the bursa has been shown in the fly *Dryomyza anilis* (Otronen 1997).

In conclusion, our study shows that in the yellow dung fly the spermathecae are not filled homogeneously during copulation, leading to different proportional representation of the second male's sperm across spermathecae of the same female. This provides a possible functional explanation for the multiple spermathecal chambers in this species. Moreover, experimental manipulation of the females after copulation indicates that muscle-dependent sperm storage processes can occur after copulation, suggesting that muscular control might also be implicated during sperm storage processes in copula. These results strongly suggest that the female reproductive tract is actively involved in sperm movement from the site of insemination to the site of storage.

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