A female nervous system is necessary for normal sperm storage in *Drosophila melanogaster*: a masculinized nervous system is as good as none

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A male *Drosophila melanogaster* deposits many more sperm in a female's bursa copulatrix than are stored in her ventral receptacle or paired spermathecae soon after copula has ended. The remaining sperm are expelled by the female. These observations suggest a sexual conflict over the processes involved in sperm storage. We used genetically manipulated flies to study the role of the central nervous system in sperm storage. Flies with female bodies but masculinized nervous systems, or isolated female abdomens, stored significantly fewer sperm than did control females. Furthermore, compared with control flies, there were relatively more sperm in the ventral receptacle and relatively fewer in the spermathecae. These results suggest that the female nervous input counteracts the male's attempts to force sperm into the ventral receptacle during copula and promotes active transport of sperm to the spermathecae during and after copula. The female is clearly a very active partner in influencing processes involved in sperm competition, especially as only stored sperm can be used later to fertilize eggs. To our knowledge, this is the first study to show directly the involvement of the female nervous system in sperm storage.

Keywords: Diptera; insemination; reproduction; sex; sexual conflict; sperm transfer

1. INTRODUCTION

The details of the processes involved in sperm storage are little understood (Eberhard 1996), and there is likely to be conflict between mating partners about storage, e.g. over how many sperm from a particular male a female should store. A male's reproductive success will clearly be increased if a female stores his sperm; otherwise the sperm may simply be expelled and not used to produce offspring. As females of many species mate repeatedly, selection may favour females that do more than simply fill their sperm stores. For example, by mating with several males and having sperm available from each, a female could increase the genetic variability among her offspring or could exercise cryptic choice among her partners (Thornhill 1983; Eberhard 1996; Hosken & Stockley 1998).

Sperm competition and sexual conflict during mating are well established in *Drosophila melanogaster* (Boorman & Parker 1976; Gromko *et al.* 1984; Rice 1996). During a single copula, a male transfers a few thousand sperm to the female (Gilbert 1981), even though far fewer are subsequently stored in the female's ventral receptacle and paired spermathecae (Gromko *et al.* 1984). The anatomy of the female reproductive system is such that sperm are deposited in the bursa copulatrix (sometimes called the

uterus), close to the entrances of the ventral receptacle and the spermathecal ducts (Gromko *et al.* 1984). It is conceivable that the male could force sperm into the ventral receptacle, as the opening of his genitalia is very close to the entrance during copula, but less likely that he could force them all the way up the ducts and into the spermathecae. In other species, active female participation seems to be required to move sperm to the spermathecae. Linley & Simmons (1981) argue that a female fly must pump fluid out of her reproductive system, probably into the haemolymph, to allow sperm to move up the system. Villavaso (1975) showed in a beetle that female muscular activity is necessary to displace already stored sperm.

Using genetically manipulated flies, we examined the role of the central nervous system (CNS) in the process of sperm storage soon after copula. We compared the storage patterns of wild-type females with those of females with a masculinized CNS, or of isolated female abdomens without a CNS.

2. THE FLIES

In *Drosophila*, sex is determined by the ratio of X chromosomes to sets of autosomes. This primary signal initiates a short cascade of regulatory genes of which the differential activity dictates female or male sexual development. One of these regulatory genes is *transformer*

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Table 1. *Genotypes and phenotypes of female flies resulting from the cross of y/y; Df(3L)st^{j7}, st tra¹ Ki roe p^p hs[tra-fem]/TM6, tra⁺ females with w/Y; th st tra¹ cp ri p^p/TM6, tra⁺ males*

symbol	genotype ^a	distinguishing phenotype	sexual phenotype at 25 °C	sexual phenotype with heat shocks
X1	X/X; tra⁺/st tra¹ p^p Ki hs[tra-fem]	kinked (Ki) bristles	normal female, fertile	normal female, fertile
X2	X/X; st tra¹ p^p/st tra¹ p^p Ki hs[tra-fem]	kinked (Ki) bristles, light orange eyes (st p ^p)	'female' with male CNS, male behaviour, sterile	normal female, female behaviour, fertile
X3	X/X; tra⁺/tra¹	like wild type	normal female, fertile	normal female, fertile
Y2	X, y/Y; st tra¹ p^p/st tra¹ p^p Ki hs[tra-fem]	yellow cuticle (y), kinked bristles (Ki), light orange eyes (st p ^p)	'female' with male CNS, male behaviour, sterile	female, female behaviour, sterile (no oogenesis with XY germ line)

^a Only the diagnostically relevant markers are given (y, Ki, st p^p); sex-chromosome constitution and the situation for *tra* function are in bold print.

(*tra*). When *tra* is active, animals develop as females; when it is inactive, male development ensues (see Cline & Meyer (1996) for review). *X/X* flies that are homozygous for the recessive mutation *tra¹* develop as sterile pseudo-males that display the full repertoire of male courtship (Hall *et al.* 1994). A transgene, *hs[tra-fem]*, carrying the female-specific cDNA of *tra* under control of a heat-shock promoter, restores the female phenotype of *X/X; tra¹/tra¹* flies when raised at 25 °C and also transforms their XY siblings into phenotypic females (pseudofemales) (McKeown *et al.* 1988). Both types of 'females', *X/X; tra¹/tra¹ hs[tra-fem]* (referred to as X2) and *X/Y; tra¹/tra¹ hs[tra-fem]* (referred to as Y2), however, show male courtship behaviour. For a fly to show male courtship behaviour, certain regions of the CNS must be male (Hall 1977; Greenspan 1995). Thus, in our X2 and Y2 flies at least the neural centres required for sexual behaviour are male, probably as a result of the insufficient expression of the transgene in the CNS at 25 °C. Oogenesis is normal in X2 'females', but they are unable to lay eggs, presumably owing to their masculinized nervous system. In addition, our flies possess the male-specific muscle MOL, which is present only when the motor neurons innervating the myoblasts of the fifth abdominal segment are male (Lawrence & Johnston 1986). All of these male characters disappear when the expression of the transgene is forced by applying a series of heat shocks during a critical period of development. Specifically, male sexual behaviour is abolished and is replaced by female reproductive behaviour, and X2 flies become fertile. Studies with genetic mosaics have revealed that certain parts of the nervous system must be female if a fly is to perform female reproductive behaviour (Tompkins *et al.* 1983). Therefore, these parts of the nervous system of our flies must be feminized after repeated heat shocks. Full details of the reproductive biology of our flies will be published elsewhere (Arthur & Nöthiger 1998).

Flies were raised on standard food. Experimental (X2 and Y2) and control flies (referred to as X1 and X3) were generated by crossing *y/y; Df(3L)st^{j7}, st tra¹ Ki roe p^p hs[tra-fem]/TM6, tra⁺ females to *w/Y; th st tra¹ cp ri p^p/TM6, tra⁺ males* (for gene symbols, see Lindsley & Zimm (1992)). This cross generates four identifiable 'female' phenotypes (table 1).*

Animals were subjected to two different temperature regimens during development. The first group was raised

at 25 °C. With this treatment, X2 and Y2 develop as 'females' with a masculinized nervous system; X1 and X3 become normal females, as a result of a wild-type copy of *tra⁺*. The second group was subjected to repeated heat shocks at 34 °C for 30 min every 8 h from oviposition until eclosion of the adult flies. The heat shocks lead to strong expression of the female-determining transgene *hs[tra-fem]* also in the CNS, so that X2 and Y2 now develop as females with a female CNS, displaying female behaviour. Comparisons of heat-shocked and non-heat-shocked X1 and X3 control flies allowed us to monitor any effects of the heat shocking itself or of the presence of the *hs[tra-fem]* construct.

In addition to these flies, we used normal females and isolated female abdomens from an Oregon-R wild-type stock.

3. EXPERIMENTAL METHODS

A female fly or a detached female abdomen and a wild-type male were placed in an observation chamber. Only those pairs that had stayed in copula for at least 14 min were used in the analyses. The females were then dissected 60–75 min after copula. We confirmed that in all flies the bursa copulatrix was full of sperm, indicating that normal sperm transfer had occurred. A pilot study had shown that it was sufficient to wait 1 h after copula to permit normal sperm storage in our Oregon-R strain, confirming earlier results by Fowler (1973). Sperm were counted in the ventral receptacle and in the two spermathecae, using the DAPI staining method for nuclei (Bressac & Hauschteck-Jungen 1996).

We first analysed the total number of sperm stored by each female. We then examined the distribution of sperm among storage organs by comparing the proportion in the ventral receptacle with that in one of the spermathecae. As these values are not independent, we used repeated-measures analysis of variance to examine the effects (von Ende 1993). We used the arcsin square-root transformed values of the proportions in the statistical analysis, as recommended by Sokal & Rohlf (1981). As the spermathecae were removed without noting which had been on which side of the female, they were classified within each female simply by the number of sperm they contained, and the one with the larger number was used in the analysis below. This ensured there was an unexamined degree of freedom for the proportions of sperm in each storage organ in the repeated-measures analysis of variance below. If there was no unexamined

Table 2. *The numbers of sperm counted in the three storage organs for each genotype and treatment*

(HS denotes the heat-shocked groups. Ore-R refers to the Oregon-R stock.)

genotype	ventral	receptacle	spermatheca 1		spermatheca 2		<i>N</i>
	mean	s.e.	mean	s.e.	mean	s.e.	
Ore-R abdomens	78.0	26.3	2.0	1.1	0.0	0.0	19
X2	99.7	25.2	2.8	0.5	0.6	0.3	27
Y2	66.7	13.3	4.1	1.1	1.6	0.8	29
intact Ore-R	376.6	3.6	91.3	1.0	80.8	4.3	20
X2 HS	381.5	4.4	90.3	1.6	82.9	1.8	15
Y2 HS	363.4	4.7	80.5	1.3	72.8	1.3	15
X1 HS	386.0	2.7	89.6	1.6	77.2	5.7	15
X3 HS	386.5	2.4	87.5	1.5	79.7	10.9	15
X1	387.7	10.8	85.9	1.9	79.6	1.8	15
X3	385.8	2.5	85.9	1.8	75.3	5.3	15

Table 3. *The analysis of variance results for (a) the total numbers of sperm stored and (b) their distribution among the storage organs*

(Genotypes or only abdomen is referred to as treatment and the experimental and control groupings as sets.)

source of variation	d.f.	mean square	<i>F</i>	<i>p</i>
<i>(a) Total numbers of sperm stored</i>				
sets	1	2285286.10	614.90	<0.001
treatment within set	8	3716.49	0.69	0.696
error	175	5349.88	—	—
<i>(b) Distribution of sperm between receptacle and spermatheca</i>				
sets	1	0.11	196.12	<0.001
treatment within set	8	0.00055	1.04	0.408
between-subjects error	165	0.00053		
site (receptacle or spermatheca)	1	12.77	107.03	<0.001
set by site	1	0.95	29.97	0.001
treatment within set by site	8	0.03	0.27	0.976
within-subjects error	165	0.12	—	—

degree of freedom, error terms could not be calculated for the within-subjects effects. The proportions were examined because we were interested in the relative amounts of sperm in the different storage organs. If the actual counts had been used, the very different mean numbers of sperm stored by flies in the control and experimental groups (see below) would result in statistically significant effects being generated simply by the same relative change across the storage organs.

There were ten treatments in the analysis: Oregon-R females; Oregon-R isolated female abdomens; and X1, X2, X3 and Y2 genotypes, which were either heat shocked or not heat shocked. The non-heat-shocked X2 and Y2 genotypes and the abdomens were considered to be experimental treatments, as these flies had either a masculinized or no CNS. The other genotypes were control treatments. These two groups are referred to as sets, in the analyses below.

4. RESULTS

First, the total numbers of sperm in the storage organs were analysed. The treatments were nested within the experimental or control sets. The experimental flies stored significantly fewer sperm than the control flies, but

there was no separate effect of the interaction among the treatments (tables 2 and 3*a*). This latter result means that there were no significant differences among the treatments within the two sets, most importantly that the detached abdomens were little different from the non-heat-shocked X2 and Y2 experimental 'females'.

The distribution of sperm among the storage organs was examined using nested repeated-measures analysis of variance on the transformed proportions of the sperm stored in the ventral receptacle and the spermatheca with the larger number of sperm. There was a highly significant interaction between the sets and the storage organs, but no separate effect of the interaction between the treatments and the storage organs (table 3*b*). Figure 1 shows that, compared with the control flies, our experimental flies stored relatively more sperm in the ventral receptacle and relatively fewer in the spermatheca.

No significant differences were found when comparing only X1 and X3 flies for the effect of the heat shocks alone, either on the total number of sperm or on their distribution among the storage organs (table 4). The storage organs contained very different proportions of sperm, but these differences were the same as in the other

Figure 1. The pattern of sperm stored by experimental flies (filled symbols) and control flies (open symbols). Means are given with their standard errors (the standard errors of the control flies in the graph are so small that they are concealed by the symbols).

control treatments in figure 1; there were many more sperm in the ventral receptacle than in the spermatheca.

5. DISCUSSION

Our results show that the female nervous system is substantially responsible for controlling the process of sperm transport from the bursa copulatrix to the storage organs. Although all our flies had many hundreds of sperm in the bursa, the control flies stored many more sperm, in total, than did the experimental flies with masculinized or disconnected nervous systems. It is likely that the masculinized nervous systems of our experimental females gave no instructions to store sperm, as a

male nervous system has not been selected to control sperm storage. In support of this interpretation, there was very little difference between the isolated abdomens of Oregon-R females disconnected from the main input of the female nervous system, and the X2 and Y2 'females' raised at 25 °C.

Heat shocking, the method used to boost expression of the feminizing transgene, had in itself no significant effect on sperm storage in the control X1 and X3 females. This result shows that it was indeed the low or high expression of *tra* that was responsible for the differences between our experimental and control flies.

There was also a difference between the experimental and control flies in the distribution of sperm among the storage organs. There are two possibilities for sperm transfer to the ventral receptacle. The more straightforward explanation is that sperm are first transported there (Fowler 1973), and as experimental females retained many fewer sperm they therefore had proportionately fewer to move to the spermathecae. However, this does not seem to be the case. Fowler *et al.* (1968) found when between 51 and 100 sperm were stored, i.e. the range including the mean number in our experimental 'females', 65.6% were in the receptacle—a much smaller proportion than the 87.5% in our experimental flies. This contrasts with the similar proportions of 73.2% (Fowler *et al.* 1968) and 69.7% (our data) in the ventral receptacles in the 501–600 range. The mean of our control flies lies in this range. Our experimental flies thus had higher proportions of sperm in their ventral receptacles than would be expected from the numbers of sperm stored.

The second possibility is that the male normally attempts to force sperm into the ventral receptacle during copula, which is plausible as the sperm are deposited there (Gromko *et al.* 1984), probably because the male genitalia are very close to the entrance of the ventral receptacle during copula. Our control females could have had lower proportions of sperm there because they did not cooperate with, or even directly resisted, the male's attempts to fill their storage organs. Perhaps the entrance

Table 4. *The analysis of variance results for the test of the effect of the heat shocks alone for (a) the total numbers of sperm stored and (b) their distribution among the storage organs (i.e. using only X1 and X3 flies)*

source of variation	d.f.	mean square	F	p
(a) Total numbers of sperm stored				
genotype	1	106.67	0.14	0.711
heat shock	1	153.60	0.20	0.657
genotype by heat shock	1	194.40	0.25	0.618
error	56	771.15		
(b) Distribution of sperm between receptacle and spermatheca				
genotype	1	0.00000008	0.02	0.899
heat shock	1	<0.00000001	<0.01	0.998
genotype by heat shock	1	0.00000556	1.06	0.308
between-subjects error	56	0.00000526		
site (receptacle or spermatheca)	1	0.10249989	23348.21	<0.001
site by genotype	1	0.00000134	0.31	0.582
site by heat shock	1	0.00000969	2.21	0.143
site by genotype by heat shock	1	0.00000001	<0.01	0.965
within-subjects error	56	0.00000439		

is relatively narrow, with the aperture under female nervous control. The female could also use the layer of visceral muscles surrounding the receptacle (Blaney 1970) and the nerve fibres between the coils of the ventral receptacle (Miller 1950) to control the filling of the organ.

It appears that female nervous input is even more important for normal sperm storage in the spermathecae; we found very few sperm in the spermathecae of our experimental flies. This would be consistent with female activity being involved in sperm storage in these organs in other species (Linley & Simmons 1981; Villavaso 1975). The spermathecae are mainly used as long-term sperm stores (Gromko *et al.* 1984) and so the difference in storage patterns between the ventral receptacle and spermathecae could have long-term consequences for a male's fertilization success, mainly due to differences in female nervous input during and soon after copula.

In addition to sperm, a male passes many substances to females during copula and these can influence the female's later behaviour (Gromko *et al.* 1984; Kubli 1996). Some of these substances may have evolved to increase a male's success in sperm competition (Harshman & Prout 1994) and we suggest this could be achieved by influencing fluid transport in the female tract.

Why females would be selected to have different mixtures of sperm from their mating partners in their different storage organs is unclear, but cryptic choice of males by the females is an intriguing possibility (Ward 1993; Eberhard 1996). Our results are, to our knowledge, the first to show directly that the nervous system is clearly involved in sperm storage. This makes it plausible that a female could compare her mating partners and use this information later when selecting sperm to fertilize eggs.

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