The Journal of Experimental Biology 206, 3521-3528

doi:10.1242/jeb.00585



# An early role for the *Drosophila melanogaster* male seminal protein Acp36DE in female sperm storage

Margaret C. Bloch Qazi* and Mariana F. Wolfner†

*Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853, USA*

*Present address: Department of Biology, Nobel Hall of Science, Gustavus Adolphus College, St Peter, MN 56082, USA

†Author for correspondence (e-mail: mfw5@cornell.edu)

*Accepted 2 July 2003*

**Summary**

**Female sperm storage is an essential component of reproduction in many animals. In insects, female sperm storage affects fecundity, sperm competition/preference and receptivity to re-mating. Female sperm storage consists of several stages, including sperm entry into the sperm storage organs (SSOs), maintenance within the SSOs and exit from the SSOs. The *Drosophila melanogaster* male seminal protein Acp36DE is essential for female sperm storage. Acp36DE associates with sperm and localizes to specific regions of the female reproductive tract, including the SSOs. We determined the stage of sperm storage at which Acp36DE acts by comparing the timing of initial sperm entry into storage as well as the rates of sperm accumulation and release from the SSOs in the presence or absence of Acp36DE. Acp36DE accelerates sperm accumulation into storage but does not mediate the entry of the first sperm into storage. This finding also demonstrates that the initial stage of sperm storage consists of multiple steps. Acp36DE enters the SSOs before sperm, and its residence within the SSOs does not require sperm. We propose that once sperm storage has initiated, Acp36DE acts as a guidance factor helping subsequent sperm move into storage, a corral concentrating sperm around the SSO entrances and/or a trigger for responses within the female that accelerate storage of sperm.**

Key words: Acp36DE, reproduction, insect, sperm storage, seminal fluid protein, sperm, *Drosophila melanogaster*.

## Introduction

Sperm storage within females is a critical component of reproduction in many animals and is a nearly ubiquitous phenomenon among insects (reviewed in Gillott, 1988; Neubaum and Wolfner, 1999a). As a physiological process, sperm storage consists of several stages, including sperm accumulation, maintenance/retention in and exit from female sperm storage organs (SSOs; Bloch Qazi et al., 2003). The mechanisms involved in these stages are, however, not well understood. Mechanical influences such as female muscular contractions, ejaculate deposition near to or within the site of sperm storage, and sperm motility can influence female sperm storage (reviewed in Bloch Qazi et al., 1998; Neubaum and Wolfner, 1999a; Simmons, 2001; Bloch Qazi et al., 2003). Furthermore, while chemical substances are known to affect sperm storage (*Rhodnius prolixus*, Davey, 1958; *Drosophila melanogaster*, reviewed in Neubaum and Wolfner, 1999a; Bloch Qazi et al., 2003), the nature of their action is largely a mystery. *D. melanogaster* provides a unique opportunity to study the molecular mechanisms of female sperm storage because a male seminal fluid protein, Acp36DE, has been identified as a major player in this process (Bertram et al., 1996; Neubaum and Wolfner, 1999b).

In *D. melanogaster*, female sperm storage is important for fitness because a female can fertilize eggs for up to 2 weeks using sperm stored from a single mating (Gilbert, 1981). Several thousand sperm are deposited into the uterus within the first half of the ~20 min copulation, and sperm storage begins before copulation ends (Lefevre and Jonsson, 1962; Tram and Wolfner, 1999; Gilchrist and Partridge, 2000). The SSOs, a pair of spermathecae and the single seminal receptacle, are located at the anterior end of the uterus. By 6 h after mating, sperm storage has leveled off at up to 1000 sperm (Gilbert, 1981; Neubaum and Wolfner, 1999b; Tram and Wolfner, 1999). Both evolutionary and quantitative studies indicate that the seminal receptacle is the primary sperm storage organ (Gilbert, 1981; Neubaum and Wolfner, 1999b; Pitnick et al., 1999).

Normal female sperm storage in *D. melanogaster* requires the transfer both of sperm from the male's seminal vesicles and of proteins (Acps) secreted from the male's accessory glands (Tram and Wolfner, 1999). Females that mated with males expressing very low levels of Acps (~1% wild-type levels) have few sperm in storage despite receiving normal quantities of sperm (Kalb et al., 1993; Tram and Wolfner,

1999), indicating that Acps are indeed needed for sperm storage. In experiments with repeated matings, Acps were required for both sperm maintenance within females and subsequent use of sperm for fertilization (Hihara, 1981; Xue and Noll, 2000).

One Acp in particular, Acp36DE (Bertram et al., 1996), is necessary for normal sperm storage (Neubaum and Wolfner, 1999b). Females mated to males lacking Acp36DE have significantly fewer sperm in storage 6 h after mating than females mated to wild-type males (Neubaum and Wolfner, 1999b). Males transferring Acp36DE have higher sperm precedence than do males who do not transfer Acp36DE, presumably as an indirect consequence of Acp36DE's mediating storage of more sperm (Chapman et al., 2000). Some alleles of Acp36DE correlate with the outcome of sperm competition (Clark et al., 1995); allelic variation could alter either the abundance of Acp36DE or the efficacy of Acp36DE's sperm storage function, thereby affecting sperm precedence patterns. However, Acp36DE does not affect sperm viability in storage (Neubaum and Wolfner, 1999b).

The localization and persistence of Acp36DE provides information about its role in female sperm storage. Acp36DE is detected in the female as early as 5 min, but not more than 6 h, after the start of mating, corresponding with the time course of sperm accumulation within storage (Gilbert, 1981; Bertram et al., 1996; Neubaum and Wolfner, 1999b; Tram and Wolfner, 1999). Acp36DE localizes to the ventral side of the oviduct wall just anterior to the openings of the SSOs (Bertram et al., 1996) and is also found at the anterior edge of the mating plug (Lung and Wolfner, 2001). Acp36DE associates with sperm in the region of the uterus closest to the SSO openings, and this association withstands *in vitro* manipulation (Bertram et al., 1996; Neubaum and Wolfner, 1999b). Finally, Acp36DE was detected in the female SSOs at 2 h after mating, when sperm storage is largely complete (Gilbert, 1981; Neubaum and Wolfner, 1999b; Tram and Wolfner, 1999).

There are several, not mutually exclusive, ways in which Acp36DE could mediate sperm storage. These include facilitating the movement of sperm into storage, organizing sperm either outside or inside the SSOs, marking the entrance to sperm storage, causing retention of sperm in storage and/or inducing or modulating female response(s) to sperm. Here, we explored the mechanism of Acp36DE's effects on sperm storage by examining the timing of these effects as well as the requirements for Acp36DE localization to the SSOs. Our results demonstrate that sperm entrance into storage and their accumulation within storage are separately controlled steps and that Acp36DE plays a role in sperm accumulation only. Furthermore, the early and sperm-independent localization of Acp36DE in the SSOs suggests that Acp36DE might function from within the female SSOs. We therefore suggest that Acp36DE could act as a sperm guidance factor, as a corralling substance and/or to stimulate the female to store sperm efficiently.

## Materials and methods

### *Fly strains*

Flies (*Drosophila melanogaster* Meig) were raised on standard yeast–glucose media at 25°C and a 12 h:12 h L:D cycle. Female flies were collected within 6 h of eclosion to ensure virginity. Males were collected 24–48 h after eclosion. All experiments were conducted on flies that were 3–6 days post eclosion. Males from three genotypes (Neubaum and Wolfner, 1999b) were used to test the effects of Acp36DE on sperm storage: (1) *Acp36DE$^{1}$/Df(2L)H20* males, which transfer normal quantities of sperm and seminal fluids but no Acp36DE, (2) *Acp36DE$^{1}$/CyO* control males and (3) *Acp36DE$^{+}$/Df(2L)H20* control males. Two different lines were used as controls for the genetic background of the Acp36DE-deficient males: *Acp36DE$^{1}$/CyO* controlled for the background of the mutagenized line, and *Acp36DE$^{+}$/Df(2L)H20* controlled for the background of the deficiency line as well as for effects of loci other than Acp36DE uncovered by the deficiency (see Neubaum and Wolfner, 1999b for additional details). Spermless males (sons of Canton S males × *bw sp tud$^{1}$* females; Boswell and Mahowald, 1985) were produced as in Bertram et al. (1996). A transgene possessing the coding sequences for green fluorescent protein (GFP) driven by the sperm-specific *don juan* (*dj*) promoter (Santel et al., 1997) inserted on the X chromosome (strain provided by B. Wakimoto) was crossed into the *Df(2L)H20/CyO* strain. This allowed us to generate Acp36DE-deficient or control males, as above, whose GFP-labeled sperm could be visualized within the female reproductive tract. When crossed into the *Acp36DE$^{1}$* or *Acp36DE$^{+}$* background, males produced GFP-labeled sperm and were either deficient or wild-type, respectively, for Acp36DE. Wild-type females were either Oregon R or Canton S, as stated. We saw no significant difference between the two types of females in the number of sperm stored 6 h after mating ($t_{16}$=0.53, $P$=0.61). Because these females have a similar sperm storage pattern, using them in separate experiments should not affect the observed trends.

### *Timing of sperm storage in females*

Effects of Acp36DE on sperm storage were examined by counting sperm stored within female SSOs (seminal receptacles and spermathecae) at various times after the start of mating. Virgin Oregon R females were individually paired with an *Acp36DE$^{1}$/Df(2L)H20* or *Acp36DE$^{1}$/CyO* male in a vial containing food. At 0.3, 0.5, 0.7, 1, 2, 6, 10, 24, 48 or 72 h after the beginning of mating, the female was removed and processed for sperm counts as described in Neubaum and Wolfner (1999b). Blindly coded slides of individual female SSOs were examined for the presence of orcein-stained sperm at 100× magnification using a compound microscope with transmitted light (Zeiss Axioskop). Each sample was counted twice. Variation among repeated counts of the same sample was, on average, 8% of the sample mean, indicating consistency among individual sample counts ($N$=241 samples).

In separate experiments designed to compare the initial timing of Acp36DE entry with sperm entry into the SSOs,

Table 1. *Observations of sperm presence within female seminal receptacles at 0.25 h and 0.33 h after the start of mating*

| Time after the start of mating | Male genotype | Acp36DE present? | Sperm present (*N*) | Sperm absent (*N*) | % cases of sperm storage |
|---|---|---|---|---|---|
| 0.25 h | *Acp36DE$^1$/CyO* | + | 12 | 2 | 85.7 |
| | *Acp36DE$^1$/Df(2L)H20* | – | 3 | 10 | 23.1 |
| | *Acp36DE$^+$/Df(2L)H20* | + | 0 | 4 | 0 |
| 0.33 h | *Acp36DE$^1$/CyO* | + | 13 | 1 | 92.9 |
| | *Acp36DE$^1$/Df(2L)H20* | – | 9 | 3 | 75.0 |
| | *Acp36DE$^+$/Df(2L)H20* | + | 4 | 3 | 57.1 |

matings between Canton S females and males (*N*=23) were interrupted 10 min after the start of copulation, and sperm storage was quantified as described above.

The significance of differences in mean number of sperm stored both at different times and between females mated to males with or without Acp36DE was tested using a two-factor analysis of variance (ANOVA). Linear contrasts were performed as planned comparisons to examine increases in sperm storage within 6 h of the start of mating for each type of male (Neter et al., 1996). A least-square means contrast tested the null hypothesis that a linear combination of group parameters (corresponding to each time point examined) was equal to zero. The means and S.E.M.s used were calculated from the time effect in the ANOVA analysis. *t*-tests were used to determine at which times mean female sperm storage differed depending on the presence of Acp36DE. The depletion of sperm from female storage for each type of male (time points after 6 h) was modeled using regression analysis, and a *t*-test of the slope coefficients (b) tested whether the slopes were homogeneous. All sperm counts were transformed [$\sqrt{(\text{value} + 1)}$] to meet the assumptions of parametric statistical tests, but untransformed data are used in figures. Statistical analysis was performed using StatView software or JMP (both SAS Inc., Cary, NC, USA).

### *Acp36DE presence and persistence in the SSOs*

Wild-type (Canton S) females were mated to wild-type (Canton S) or spermless (*tudor*-progeny; see Materials and methods) males. Pairs were then separated to prevent re-mating. At 0.17, 0.33, 1, 10 and 48 h after the start of mating, females were dissected in Yamamoto's saline (Stewart et al., 1994). Triplicate samples of 30 seminal receptacles and spermathecae per treatment and time point were placed separately into 10 μl of protease-inhibiting buffer (Monsma and Wolfner, 1988), homogenized, processed and analyzed by western blotting as in Bertram et al. (1996).

### *Visualizing sperm storage in real time*

Effects of Acp36DE on sperm entry into the seminal receptacle and their motility therein were examined by visualizing GFP-labeled sperm stored within females that had mated to males with or without Acp36DE. Copulations were interrupted at either 0.25 h or 0.33 h after the start of mating, and females were immediately placed on ice. A female's entire reproductive tract was removed as a unit and mounted in 4% methyl cellulose (Sigma, St Louis, MO, USA) in Yamamoto's saline. Optical sections of reproductive tracts were imaged at 40× (Zeiss Axiovert 10) then reassembled for analysis (BioRad MRC 600). Only those samples in which sperm were observed in the uterus (indicating sperm transfer) were included in the analysis. The presence and orientation of sperm within the seminal receptacle was observed and the association between sperm presence in the seminal receptacle and male genotype was tested using a $\chi^2$ test of independence (Sokal and Rohlf, 1995).

## Results

### *Initial sperm entry into the seminal receptacle is not facilitated by Acp36DE*

The previously demonstrated positive effect of Acp36DE on the number of sperm stored by 6 h after mating (Neubaum and Wolfner, 1999b) could be due to Acp36DE's effects on the timing of initial sperm entry into storage, on the speed of sperm accumulation within storage once storage has begun and/or on prevention of premature sperm release from storage. If Acp36DE affects the timing of initial entry of sperm into storage, then sperm storage should begin earlier when females receive Acp36DE than when they do not. We examined the timing of initial sperm entry into the seminal receptacle using *dj-GFP* males that made or lacked Acp36DE. Although *dj-GFP* males transfer and store fewer sperm than wild-type males (Price, 1999), those sperm do get stored and their GFP fluorescence allowed us to detect even very low numbers of sperm as well as to directly observe their orientation and movements within the SSOs *in vivo*. At 0.25 h post-mating, sperm are already visible within storage in some females, but sperm presence or absence did not correlate with the presence or absence of Acp36DE (Table 1). Although an effect of male genotype on frequency of stored sperm was detected at this time ($\chi^2_2$=14.90, $P$<0.001), this effect was correlated with male strain background rather than with Acp36DE status. Sperm were seen more frequently in the seminal receptacles of females mated to *Acp36DE$^1$/CyO* males than in mates of either *Acp36DE$^1$/Df(2L)H20* or *Acp36DE$^+$/Df(2L)H20* males. When the analysis was repeated between *Acp36DE$^1$/Df(2L)H20* and *Acp36DE$^+$/Df(2L)H20* males only, no difference in the frequency of stored sperm was detected ($\chi^2_1$=1.12, $P$=0.29;

Table 1). The apparent effect of background might be due to the effects of hemizygosity for loci uncovered by *Df(2L)H20* or to dominant effects from loci on the *CyO* balancer. By 0.33 h after the start of mating, sperm presence in the seminal receptacles did not differ among male genotypes ($\chi^2_2$=3.72, *P*=0.155; Table 1), and 79% of all seminal receptacles (*N*=33) contained sperm. These results indicate that Acp36DE does not determine the timing of entry of the first sperm into storage.

Presence or absence of Acp36DE also did not have a noticeable effect on sperm orientation or motility. When few sperm are in storage, they often appeared disorganized, regardless of whether or not Acp36DE was present. This suggests that the observation of Neubaum and Wolfner (1999b) of disorganized sperm in storage within females not receiving Acp36DE reflected simply the small number of sperm in storage rather than any direct effect of Acp36DE. Vigorous sperm movements within the seminal receptacle were observed both when Acp36DE was present and when it was absent (data not shown).

### *Acp36DE promotes the early accumulation of sperm within SSOs*

Since the presence or absence of Acp36DE did not affect the timing of initial sperm entry into the seminal receptacle, we examined the effects of Acp36DE on the number of stored sperm in the SSOs at time points corresponding to later stages of sperm storage, including sperm accumulation and retention within storage. With and without Acp36DE, sperm accumulation in storage reaches maximal numbers by 1 h after the start of mating (Fig. 1A). The time course of accumulation, however, was influenced by the presence of Acp36DE. As reported by Neubaum and Wolfner (1999b), females receiving Acp36DE stored significantly more sperm than did females not receiving Acp36DE (two-factor ANOVA: $F_{1,207}$=28.44, *P*<0.0001). The effects of Acp36DE in promoting sperm storage were observed soon after mating had ended. By 0.5 h after the start of mating, females receiving Acp36DE stored 2.5-fold more sperm than females not receiving Acp36DE ($t_{19}$=3.42, *P*<0.003). No difference in sperm storage in the presence and absence of Acp36DE was detected at 0.3 h after the start of mating ($t_{20}$=0.676, *P*=0.507). The difference in accumulation of sperm in storage between the two male genotypes is not attributable to the effects of *Df(2L)H20*. At 1 h after the start of mating, sperm storage in females mated to control males (*Acp36DE*$^+$*/Df(2L)H20*: mean ± 1 S.E.M.=353.7±29.56 sperm) is similar to that of females mated to *Acp36DE*$^1$*/CyO* control males (Bonferroni-Dunn *post-hoc* test: *P*=0.97, *N*=25) and is significantly higher than that of females mated to *Acp36DE*$^1$*/Df(2L)H20* males (*P*=0.018, *N*=25).

This difference in the number of sperm stored in the presence or absence of Acp36DE reflects a difference in their rate of accumulation of stored sperm. When females received Acp36DE (*Acp36DE*$^1$*/CyO* mates), the number of stored sperm increased nearly threefold between 0.3 h and 0.5 h after the start of mating (linear contrast, *t*-ratio=–5.66, d.f.=62, *P*<0.0001), but the increase in number of stored sperm after 0.5 h (~53%) was not statistically significant (*t*-ratio=–0.734, d.f.=62, *P*=0.47). When females did not receive Acp36DE (*Acp36DE*$^1$*/Df(2L)H20* mates), the number of sperm within storage increased significantly between 0.3 h and 0.5 h (138%; *t*-ratio=–5.41, d.f.=65, *P*<0.0001) as well as between 0.5 h and 0.7 h (108%; *t*-ratio=–3.82, d.f.=65, *P*<0.001) but not at later time points (*t*-ratio=–0.80, d.f.=65, *P*=0.43) (Fig. 1A).

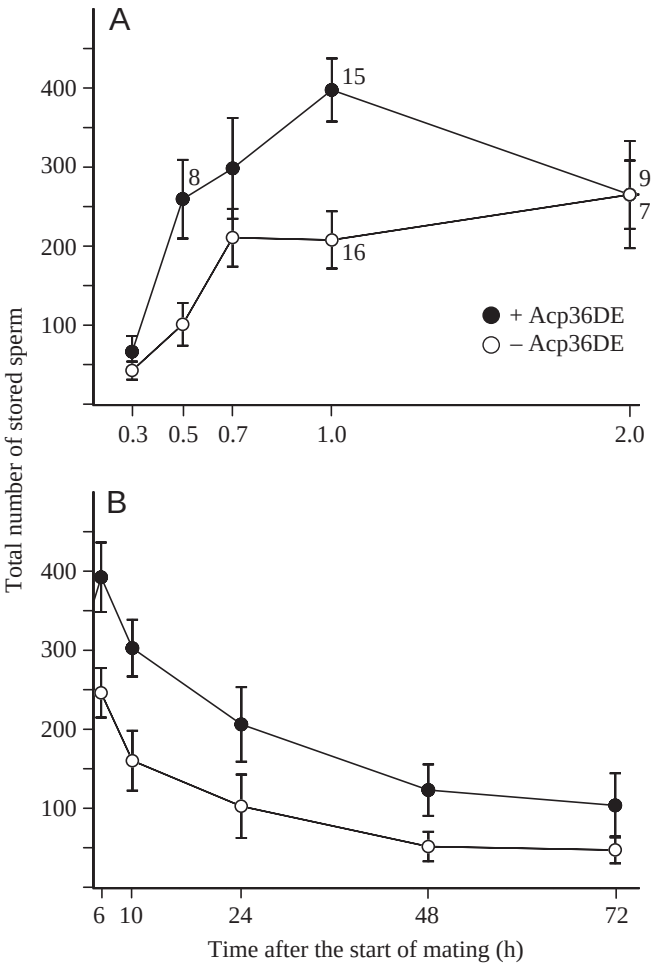


Fig. 1. (A) Mean (±1 S.E.M.) sperm storage in seminal receptacles and spermathecae at time points ranging from 0.3 h to 2 h after the start of mating for females mated to *Acp36DE*$^1$*/CyO* males (+Acp36DE; filled circles) and *Acp36DE*$^1$*/Df(2L)H20* males (–Acp36DE; open circles). Sample sizes are 11 for each mean except where indicated on the figure. (B) Sperm storage between 6 h and 72 h after the start of mating. Symbols are the same as above and *N*=11 for each point. We detected a smaller difference between the number of sperm stored in the absence of Acp36DE (relative to the control) than that reported by Neubaum and Wolfner (1999b). This discrepancy could be partially attributable to differing experimental procedures between the two studies but, in addition, modifiers affecting sperm storage may have acted to mitigate the effect(s) of the *Acp36DE*$^1$ allele during stock maintenance. Since sperm storage is an important facet of female fertility, strong selection is expected to act on sperm storage ability.

To determine if Acp36DE also influences the rate at which

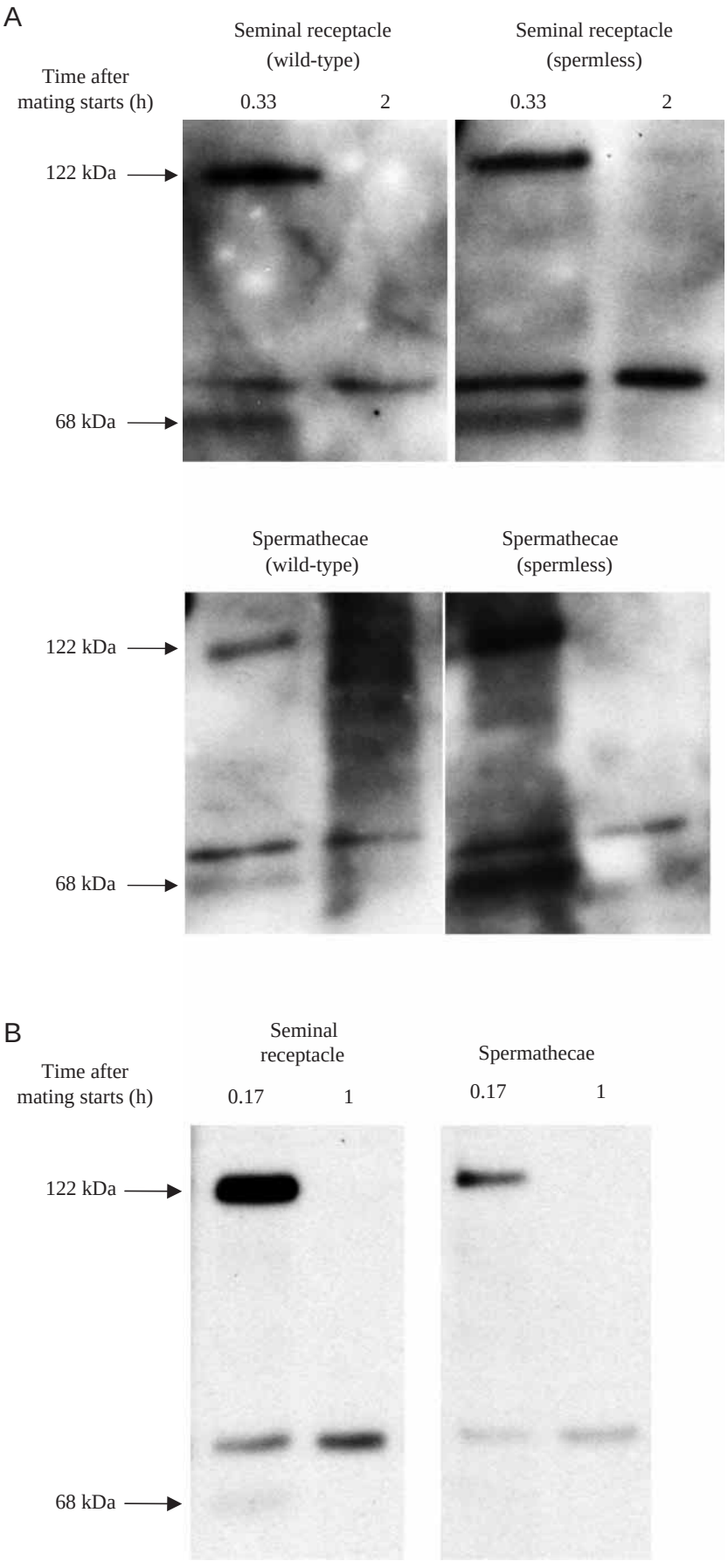


Fig. 2. (A) Full-length (122 kDa) Acp36DE and its 68 kDa processing product in the seminal receptacles (top) or spermathecae (bottom) of 90 females at 0.33 h or 2 h after the start of mating to wild-type males or to males transferring seminal fluids but no sperm. A cross-reactive protein band at 75 kDa serves as a loading control. Results were similar in repeats of this experiment. (B) Full-length (122 kDa) and processed (68 kDa; Bertram et al., 1996) Acp36DE in the seminal receptacle and spermathecae of 90 females mated with wild-type males 0.17 h or 1 h after the start of mating.

sperm exit storage, we compared the decline in numbers of stored sperm in the presence or absence of Acp36DE. The rate of sperm depletion from females receiving Acp36DE (*Acp36DE$^{1}$/CyO* males, $b$=–0.161) and females lacking Acp36DE (*Acp36DE$^{1}$/Df(2L)H20* males, $b$=–0.123) were not statistically different ($t_{30}$=0.995, 0.25<$P$<0.10), indicating that Acp36DE does not affect sperm retention (Fig. 1B).

### *Acp36DE is found in SSOs shortly after mating, and this localization does not require sperm*

To explore how Acp36DE facilitates rapid sperm accumulation, we examined its earliest detection, duration of residence and dependence on sperm for its residence in the SSOs. Acp36DE is normally found in the female SSOs and associates with sperm (Bertram et al., 1996; Neubaum and Wolfner, 1999b). To determine if Acp36DE's association with sperm is required to bring Acp36DE into the SSOs, we examined the spermathecae and seminal receptacles of females mated to wild-type or spermless males for the presence of Acp36DE. Full-length Acp36DE (122 kDa) and its 68-kDa processed form (Bertram et al., 1996) were detected in both the seminal receptacle and spermathecae at 0.33 h after the start of mating in females mated to wild-type males and in females mated to spermless males (Fig. 2A). Furthermore, a faint band corresponding to full-length Acp36DE was detected in the seminal receptacles of females mated to spermless males even 2 h after mating. Therefore, Acp36DE entry into the SSOs does not require sperm.

Since Acp36DE can enter the female SSOs in the absence of sperm transfer, does it normally enter the SSOs before sperm? To address this question, we examined the SSOs for Acp36DE at 0.17 h into mating. At this time point, all of the mating females ($N$=23) had received sperm, but sperm storage was evident in only 34.8% of

those females, each having, on average, three sperm in storage. Full-length Acp36DE (122 kDa) was already abundant in both the spermathecae and the seminal receptacles at this time (Fig. 2B) and, thus, before the entry of significant numbers of sperm into storage. Because Acp36DE is detected in the SSOs by the earliest times of sperm storage and before its effects on sperm storage are detected, Acp36DE could act from within the female SSOs to facilitate the rapid accumulation of sperm in storage.

## Discussion

Sperm storage in female *D. melanogaster* is a multi-stage process beginning with sperm entry and accumulation within storage, followed by sperm maintenance within storage and ending with sperm exiting the storage organs (reviewed in Bloch Qazi et al., 2003). Acp36DE is required for sperm storage to proceed normally (Neubaum and Wolfner, 1999b), but its stage(s) of action was not previously known. Females receiving Acp36DE from males during mating store significantly more sperm than do females that do not receive the protein (Neubaum and Wolfner, 1999b). Here, we showed that Acp36DE acts early in the sperm storage process by promoting sperm accumulation into storage. While the presence of Acp36DE does not affect the initial time of sperm entry into storage, it increases the efficiency of sperm storage twofold once sperm storage has begun. Therefore, we have also shown that initial sperm entry into storage and their accumulation within storage are separable events, controlled by different factors, one of which is Acp36DE. Effects of other, currently unidentified, loci on initial sperm entry into storage is evidenced by observed strain differences in the timing of this event in the current study. Based on counts of prepared sperm samples and observations of living sperm *in vivo* (M.C.B.Q., unpublished data), Acp36DE does not appear to facilitate sperm storage by increasing sperm motility, mediating the orientation of sperm within storage or decreasing sperm depletion from storage.

Based on our observations, we propose that Acp36DE is needed for the efficient accumulation of stored sperm after the first sperm has entered storage. Acp36DE's action is detected as early as 0.5 h after the start of mating and has consequences after sperm storage has leveled off and egg laying has begun (~1.5–3.0 h; Heifetz et al., 2000; Chapman et al., 2001). Further evidence for Acp36DE's role in rapid sperm storage comes from sperm entry and storage data for Acp36DE-control males (*Acp36DE*$^{+}$*/Df(2L)H20*). Despite a lag in initial sperm storage in females mated to *Acp36DE*$^{+}$*/Df(2L)H20* males compared with *Acp36DE*$^{1}$*/CyO* males (also controls), the mean number of sperm stored in females mated to these males was nearly the same one hour after the start of mating, and both were significantly higher than the number of sperm stored by females mated to Acp36DE-deficient males (*Acp36DE*$^{1}$*/Df(2L)H20*). Similar to other studies on sperm storage (Lefevre and Jonsson, 1962; Tram and Wolfner, 1999), sperm storage in our experiments progressed very rapidly, leveling off within 1 h after the start of mating. Because sperm entry into storage must end when an ovulated egg expels remaining unstored sperm (1.5–3 h after mating; Heifetz et al., 2000), a rapid rate of sperm accumulation within storage is essential for efficient sperm storage and subsequent female fertility.

Several, not mutually exclusive, hypotheses exist for how Acp36DE might effect the rapid accumulation of sperm in the SSOs. These include (1) acting as a factor to facilitate directed sperm movement through the uterus and into the SSOs, (2) concentrating sperm around the SSO entrances and/or (3) stimulating the female to efficiently take up sperm into her SSOs. In each of these models, sperm entry can or must be initiated by some factor other than Acp36DE.

First, Acp36DE could facilitate the directed movement of sperm or groups of sperm into storage. One mechanism by which Acp36DE could accomplish this is if its association with sperm (Neubaum and Wolfner, 1999b) promoted the 'bundling' of sperm into cords that then efficiently moved, as units, into storage. This proposed mechanism is similar to the role of the sperm apical hook in sperm train formation within the uterus of the wood mouse *Apodemus sylvaticus* (Moore et al., 2002). In *A. sylvaticus*, sperm trains have higher progressive motility than do individual sperm. In *D. melanogaster*, sperm–sperm associations could be an efficient means to effect rapid sperm accumulation within the SSOs. Another potential mechanism by which Acp36DE could facilitate directed movement of sperm into storage is suggested by Acp36DE's specific localization within portions of the female's reproductive tract. Acp36DE associates with the oviduct wall, just anterior to the SSO entrances (Bertram et al., 1996), and also the anterior end of the mating plug (Lung and Wolfner, 2001). The mating plug is a mass of congealed substances at the posterior end of the mated female's reproductive tract that confines sperm to the anterior end of the uterus, near the SSO entrances (Bairati, 1968). Perhaps Acp36DE helps to form a trellis descending from the sperm barrier in the oviduct and rising from the mating plug. Sperm, also associating with Acp36DE, could move along this trellis to reach the SSO entrances (Lung and Wolfner, 2001). A final possibility for how Acp36DE could direct sperm movement into or within storage is by helping sperm cells follow preceding sperm cells into storage in a manner formally analogous to the axon fasciculation that occurs during nervous system development. Fasciculation occurs when axons from multiple neurons follow molecular cues along a trail forged by a single pioneer axon to grow out to a target (reviewed in Tessier-Lavigne and Goodman, 1996; Van Vactor, 1999). Perhaps, Acp36DE's association with sperm (Neubaum and Wolfner, 1999b) helps subsequent sperm follow the trail of a 'pioneer sperm' into storage; although the mechanism by which this would occur is unknown.

Second, Acp36DE could concentrate sperm near the SSO entrances and thereby increase the rate of storage. Factors other than Acp36DE are already known to corral sperm. A barrier in the lower common oviduct prevents sperm (and Acps) from

moving into the oviduct (Bertram et al., 1996). When the barrier is not present (as in eggless females) sperm are displaced up the oviduct, and fewer sperm are in storage 6 h after mating (Neubaum and Wolfner, 1999b). While this barrier retains Acp36DE, Acp36DE is not necessary for barrier formation (Neubaum and Wolfner, 1999b). The mating plug also keeps sperm concentrated at the anterior end of the uterus (Lung and Wolfner, 2001).

Third, Acp36DE might stimulate sperm storage by modulating muscle contractions or changing relative fluid pressure among regions of the female reproductive tract, particularly after the first sperm has entered storage and initiated the storage process. We show that Acp36DE enters the SSOs at the earliest times of sperm storage and that its entry into the SSOs does not require sperm, suggesting that Acp36DE could act from within the SSOs to effect sperm accumulation within storage. Perhaps Acp36DE stimulates the withdrawal of fluid from the SSO lumen, resulting in a decrease of relative pressure within the SSOs. As a result of this pressure differential, sperm could be sucked into storage, as has been proposed for lower Diptera (Linley, 1981). A role for Acp36DE on the female's response to stored sperm is suggested by the finding that Acp36DE from a previous mating can facilitate female storage of subsequent-mating males' sperm (Chapman et al., 2000). This is long after Acp36DE is no longer detected in the female reproductive tract (6 h; Bertram et al., 1996).

It is not known how Acp36DE gets into the SSOs. Sperm are not needed for Acp36DE's entry into or retention in the SSOs, and Acp36DE enters the SSOs during the initial stages of sperm storage, before copulation is even complete. Since Acp36DE localizes to the oviduct wall close to the SSO entrances (Bertram et al., 1996), it may be pulled or sucked into storage by female muscular contractions, similar to the mechanism proposed for sperm entry into storage in mosquitoes (Linley, 1981). Since Acp36DE is detected in the uterus as early as 5 min after mating begins (Bertram et al., 1996) and sperm transfer usually occurs within 8–10 min of the start of mating (Gilchrist and Partridge, 2000), the mating plug may create pressure within the female reproductive tract, pushing Acp36DE anteriorly into the SSOs. Alternatively, since large amounts of Acp36DE are transferred to females, its entry into the SSOs could just be stochastic.

In conclusion, we have shown that sperm entry into and accumulation within female SSOs are distinct events controlled by different factors. Acp36DE facilitates the rapid accumulation of sperm within female SSOs, but only after the first sperm has entered storage. In addition to its association with sperm, the mating plug and the uterus, Acp36DE accumulates within the SSOs at the earliest stages of sperm storage and its accumulation there does not require sperm. These results suggest that Acp36DE could act from within the SSOs to assist them in taking up sperm efficiently. Future identification and description of the location of Acp36DE-binding proteins will help elucidate Acp36DE's role in rapid sperm accumulation within the SSOs. Since rapid storage of sperm is important for both male and female reproductive fitness, understanding Acp36DE's role in sperm storage provides novel insights into the mechanisms of male and female reproductive interactions.

Our appreciation to Sophia Cleland for preliminary experiments on Acp36DE localization in sperm storage organs with spermless males, Sanjive Qazi for statistical advice, and Carol Bayles and Yael Heifetz for help with confocal microscopy. Barbara Wakimoto generously supplied the flies with X-linked *dj-GFP*, and Thura Soe-Htwe crossed the transgene into *Df(2L)H20/CyO*. We are grateful to Shannon Albright, Vanessa Horner, Lisa McGraw, Jacob Muller, Ravi Ram and Uyen Tram for insightful and constructive comments on drafts of this manuscript. This work was supported by NIH grant HD38921 (M.F.W.) and an American Cancer Society Postdoctoral Fellowship (to M.C.B.Q.).

## References

**Bairati, A.** (1968). Structure and ultrastructure of the male reproductive system in *Drosophila melanogaster* Meig. 2. The genital duct and accessory glands. *Ital. J. Zool.* **2**, 105-182.

**Bertram, M. J., Neubaum, D. M. and Wolfner, M. F.** (1996). Localization of the *Drosophila* male accessory gland protein Acp36DE in the mated female suggests a role in sperm storage. *Insect Biochem. Mol. Biol.* **26**, 971-980.

**Bloch Qazi, M. C., Aprille, J. R. and Lewis, S. M.** (1998). Female role in sperm storage in the red flour beetle, *Tribolium castaneum*. *Comp. Biochem. Physiol. A* **120**, 641-647.

**Bloch Qazi, M. C., Heifetz, Y. and Wolfner, M. F. (**2003). The developments between gametogenesis and fertilization: ovulation and female sperm storage in *Drosophila melanogaster*. *Dev. Biol.* **256**, 195-211.

**Boswell, R. E. and Mahowald, A. P.** (1985). *tudor*, a gene required for assembly of the germ plasm in *Drosophila melanogaster*. *Cell* **43**, 97-104.

**Chapman, T., Neubaum, D. M., Wolfner, M. F. and Partridge, L.** (2000). The role of male accessory gland protein Acp36DE in sperm competition in *Drosophila melanogaster*. *Proc. R. Soc. Lond. B. Biol. Sci.* **267**, 1097-1105.

**Chapman, T., Herndon, L. A., Heifetz, Y., Partridge, L. and Wolfner, M. F.** (2001). The Acp26Aa seminal fluid protein is a modulator of early egg hatchability in *Drosophila melanogaster*. *Proc. R. Soc. Lond. B. Biol. Sci.* **268**, 1647-1654.

**Clark, A. G., Aguadé, M., Prout, T., Harshman, L. G. and Langley, C. H.** (1995). Variation in sperm displacement and its association with accessory gland protein loci in *Drosophila melanogaster*. *Genetics* **139**, 189-201.

**Davey, K. G.** (1958). The migration of spermatozoa in the female of *Rodnius prolixus* Stal. *J. Exp. Biol.* **35**, 694-701.

**Gilbert, D. G.** (1981). Ejaculate esterase 6 and initial sperm use by female *Drosophila melanogaster*. *J. Insect Physiol.* **27**, 641-650.

**Gilchrist, A. S. and Partridge, L.** (2000). Why it is difficult to model sperm displacement in *Drosophila melanogaster*: the relationship between sperm transfer and copulation duration. *Evolution Int. J. Org.* **54**, 534-542.

**Gillott, C.** (1988). Arthropoda-Insecta. In *Reproductive Biology of the Invertebrates*, vol, III, *Accessory Sex Glands* (ed. K. G. Adiyodi and R. G. Adiyodi), pp. 319-471. New York: John Wiley & Sons.

**Heifetz, Y., Lung, O., Frongillo, E. A., Jr and Wolfner, M. F.** (2000). The *Drosophila* seminal fluid protein Acp26Aa stimulates release of oocytes by the ovary. *Curr. Biol.* **10**, 99-102.

**Hihara, F.** (1981). Effects of the male accessory gland secretion on oviposition and remating in females of *Drosophila melanogaster*. *Zool. Mag.* **90**, 307-316.

**Kalb, J. M., DiBenedetto, A. J. and Wolfner, M. F.** (1993). Probing the function of *Drosophila melanogaster* accessory glands by directed cell ablation. *Proc. Natl. Acad. Sci. USA* **90**, 8093-8097.

**Lefevre, G. and Jonsson, U. B.** (1962). Sperm transfer, storage, displacement, and utilization in *Drosophila melanogaster*. *Genetics* **47**, 1719-1736.

**Linley, J. R.** (1981). Emptying of the spermatophore and spermathecal filling in *Culicoides melleus* (Coq.) (Diptera: Ceratopogonidae). *Can. J. Zool.* **59**, 347-356.

**Lung, O. and Wolfner, M. F.** (2001). Identification and characterization of the major *Drosophila melanogaster* mating plug protein. *Insect Biochem. Mol. Biol.* **31**, 543-551.

**Monsma, S. A. and Wolfner, M. F.** (1988). Structure and expression of a *Drosophila* male accessory gland gene whose product resembles a peptide pheromone precursor. *Genes Dev.* **2**, 1063-1073.

**Moore, H., Dvořáková, K., Jenkins, N. and Breed, W.** (2002). Exceptional sperm cooperation in the wood mouse. *Nature* **418**, 174-177.

**Neter, J., Kutner, M. H., Nachtsheim, C. J. and Wasserman, W.** (1996). *Applied Linear Statistical Models*. Fourth edition. Chicago: Irwin Press.

**Neubaum, D. M. and Wolfner, M. F.** (1999a). Wise, winsome or weird: mechanisms of sperm storage in female insects. *Curr. Top. Dev. Biol.* **41**, 67-97.

**Neubaum, D. M. and Wolfner, M. F.** (1999b). Mated *Drosophila melanogaster* females require a seminal fluid protein, Acp36DE, to store sperm efficiently. *Genetics* **153**, 845-857.

**Pitnick, S., Markow, T. and Spicer, G. S.** (1999). Evolution of multiple kinds of female sperm-storage organs in *Drosophila*. *Evol. Int. J. Org.* **53**, 1804-1822.

**Price, C. S. C.** (1999). Sperm competition between *Drosophila* males involves both displacement and incapacitation. *Nature* **400**, 449-452.

**Santel, A., Winhauer, T., Blumer, N. and Renkawitz-Pohl, R.** (1997). The *Drosophila don juan* (*dj*) gene encodes a novel sperm specific protein component characterized by an unusual domain of repetitive amino acid motif. *Mech. Dev.* **64**, 19-30.

**Simmons, L. W.** (2001). *Sperm Competition and Its Evolutionary Consequences in the Insects*. Princeton: Princeton University Press.

**Sokal, R. R. and Rohlf, F. J.** (1995). *Biometry: The Principles and Practice of Statistics in Biological Research*. Third edition. New York: W. H. Freeman and Co.

**Stewart, B. A., Atwood, H. L., Renger, J. J., Wang, J. and Wu, C. F.** (1994). Improved stability of *Drosophila* larval neuromuscular preparations in haemolymph-like physiological solutions. *J. Comp. Physiol.* **175**, 179-191.

**Tessier-Lavigne, M. and Goodman, C. S.** (1996). The molecular biology of axon guidance. *Science* **274**, 1123-1133.

**Tram, U. and Wolfner, M. F.** (1999). Male seminal fluid proteins are essential for sperm storage in *Drosophila melanogaster*. *Genetics* **153**, 837-844.

**Van Vactor, D.** (1999). Axon guidance. *Curr. Biol.* **9**, R797-R799.

**Xue, L. and Noll, M.** (2000). *Drosophila* female sexual behavior induced by sterile males showing copulation complementation. *Proc. Natl. Acad. Sci. USA* **97**, 3272-3275.