Male Seminal Fluid Proteins Are Essential for Sperm Storage in *Drosophila melanogaster*

Uyen Tram[1] and Mariana F. Wolfner

Department of Molecular Biology and Genetics, Cornell University, Ithaca, New York 14853-2703

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ABSTRACT

The seminal fluid that is transferred along with sperm during mating acts in many ways to maximize a male's reproductive success. Here, we use transgenic *Drosophila melanogaster* males deficient in the seminal fluid proteins derived from the accessory gland (Acps) to investigate the role of these proteins in the fate of sperm transferred to females during mating. Competitive PCR assays were used to show that while Acps contribute to the efficiency of sperm transfer, they are not essential for the transfer of sperm to the female. In contrast, we found that Acps are essential for storage of sperm by females. Direct counts of stored sperm showed that 10% of normal levels are stored by females whose mates transfer little or no Acps along with sperm.

DELIVERING sperm to females that will use them is only one component of a male's reproductive success. Once in the female genital tract, sperm can be discarded, used immediately, or stored for use over a period of time that can last a few days to years, depending on the organism (reviewed in Birkhead and Møller 1998; Neubaum and Wolfner 1999a). Females that remate prior to depleting their supply of sperm create an environment in which sperm from different males can compete for storage and usage. The mode of sperm transfer from the male to the female influences the possibility of sperm competition (Kotrba 1996; Birkhead and Møller 1998). Sperm can be deposited into the female as a package (spermatophore) encased in congealed male accessory gland secretions (reviewed in Mann 1984; Gillott 1988; Birkhead and Møller 1998) or as a free suspension in seminal fluid. All sperm transferred via spermatophore can be lost easily if the package is removed, either by the female or by a rival male, before sperm have moved from the package into the female's sperm storage organs (Kotrba 1996; Birkhead and Møller 1998). Sperm transferred as a free suspension incur the risk of leaking out of the female reproductive tract and of being diluted by sperm from subsequent males before being stored (Kotrba 1996; Birkhead and Møller 1998). Once stored, sperm still risk being displaced from storage by sperm and/or seminal fluid from subsequent males (Parker 1970; Smith 1984; Harshman and Prout 1994; Kotrba 1996; Price 1997). While it is clear that male accessory gland secretions play an important role in sperm transfer in animals that use spermatophores, it was not apparent whether these secretions also mediate sperm transfer or storage in animals that do not use spermatophores, such as *Drosophila melanogaster*.

The seminal fluid that is transferred together with sperm can bolster the male's reproductive success in several ways. In insects, seminal fluid induces physiological and behavioral changes in females (reviewed in Wolfner 1997 for *D. melanogaster*). Drosophila seminal fluid proteins cause females to elevate egg-laying rate and reduce receptivity toward courting males (Chen *et al.* 1988; Kalb *et al.* 1993; Herndon and Wolfner 1995), thus maximizing sperm usage and minimizing the chance of sperm competition occurring. In other insects, components of the seminal fluid also act as an antiaphrodisiac (Mann 1984) to discourage other males from courting the mated female, can be absorbed by the female for use in the production of eggs (Mann 1984; Pitnick *et al.* 1997), and may provide nourishment and protection for sperm (Mann 1984).

D. melanogaster males transfer several thousand sperm during mating (Kaufman and Demerec 1942; Gilbert 1981). Upon deposition in the female genital tract, sperm are rapidly sequestered to the sperm storage organs. The seminal receptacle is a long, coiled organ that branches from the posterior end of the common oviduct (Miller 1950). It is the organ that first fills with sperm. It houses 80–90% of the stored sperm (Fowler *et al.* 1968). The spermathecae are sac-like and are located more posteriorly than the seminal receptacle (Miller 1950). They are connected to the anterior end of the uterus and are believed to be long-term sperm storage organs (Gilbert 1981). Storage of sperm begins immediately after transfer to the female, but as noted by Gromko *et al.* (1984), "the length of time required for maximum sperm storage is variable (15 min to 9.4 hr)."

A role for accessory gland secretions in *D. melanogaster*

Corresponding author: Mariana F. Wolfner, Department of Molecular Biology and Genetics, 423 Biotechnology Bldg., Cornell University, Ithaca, NY 14853-2703. E-mail: mfw5@cornell.edu

[1]*Present address:* Department of Biology, University of California, Santa Cruz, CA 95064.

sperm management (transfer from male to female, sperm storage and competition in females) was first suggested from experiments in which males were mated several times in quick succession. These males became temporarily sterile (Lefevre and Jonsson 1962; Hihara 1981). Microscopic examination revealed that such males still contained mature sperm, but their accessory glands appeared deflated, suggesting that mating had depleted the males of accessory gland secretions but not mature sperm. Lefevre and Jonsson concluded from those data that males are unable to transfer sperm without accessory gland secretions. In contrast, Hihara argued that males depleted of accessory gland secretions could still transfer sperm because he observed sperm in the females mated to them. However, neither study could conclusively determine the role of accessory gland secretions in sperm transfer, since their conclusions were based on correlative evidence between the fertility of females and the appearance (fullness/emptiness) of the accessory glands of the males mated to them. Two subsequent studies also suggested that accessory gland secretions affected sperm, but again, the process that was affected was not determined. Harshman and Prout (1994) reported that secretions from the male's accessory glands can displace sperm that are resident in the female's sperm storage organs. The degree of sperm displacement varied from male to male and was correlated with allelic variation at one accessory gland protein (*Acp*) gene, while the ability to resist being displaced was correlated with allelic variation at four different *Acp* genes (Clark *et al.* 1995).

Kalb *et al.* (1993) reported that mates of transgenic males (DTA-D males) with greatly reduced accessory gland main cell secretions stored fewer sperm than females mated to control males. This indicates that Acps play a role during some stage of sperm management. However, their data did not distinguish among several hypotheses—that the transgenic males made or transferred fewer sperm, or that normal numbers of sperm were made and transferred but their storage was compromised because of the lack of Acps (Kalb *et al.* 1993).

To clarify the role of accessory gland secretions in sperm transfer and sperm storage in *D. melanogaster*, we use these transgenic DTA-D males to examine the quantity of sperm transferred by males and the number of sperm stored by females in the near absence of these secretions. We show here that Acps are essential for sperm storage but are not required for sperm transfer.

MATERIALS AND METHODS

Flies: Fly stocks were maintained on standard yeast-glucose media at 23° ± 2° on a 12:12 hr light:dark cycle. Females were from the Oregon-R wild-type stock. Males were from transgenic stocks. Males deficient in accessory gland function were generated by directed cell ablation, by expressing subunit A of diphtheria toxin in accessory gland main cells via the promoter of accessory gland gene Acp95EF (Kalb *et al.* 1993). Expression of main-cell-derived Acps is undetectable in male flies expressing the wild-type toxin. Unfortunately, these males produce no sperm, presumably because of low-level expression of the Acp95EF-toxin fusion in spermatogenic cells, killing those cells (Kalb *et al.* 1993). Therefore, for this study we used DTA-D flies, which express a mutated form of diphtheria toxin that is 100-fold less active than normal (Wilson *et al.* 1990). DTA-D males produce Acps at 1% of the level of control males (Kalb *et al.* 1993). They produce sperm (Kalb *et al.* 1993), but are poorly fertile (U. Tram and M. F. Wolfner, unpublished data). The control flies for experiments involving DTA-D males were pWgHL811.2, a transgenic but otherwise normal strain (Kalb *et al.* 1993).

Quantifying sperm: We used three different techniques to quantify sperm production, sperm transfer, and sperm storage. Direct counts were made on the number of sperm produced and stored because it was possible to do so accurately. Sperm found in the female sperm storage organs were stained with orcein, following the classical methods of Lefevre and Jonsson (1962) and Gilbert (1981). To quantify sperm production in male seminal vesicles, we used the fluorescent dye DAPI to label sperm because individual sperm heads were not distinguishable using orcein in our preparations. In comparing sperm transfer, we needed a method that allowed us to quantify sperm in the uterus, where they are present in a tangled mass. Thus, we developed a molecular technique for counting sperm that used PCR on a sperm-specific marker.

Comparing sperm production in males: To assess the production of mature sperm, we compared the number of sperm present in the seminal vesicles of 4-day-old males (see Figure 1a for a diagram of the male reproductive tract). DTA-D and control males were collected on ice within 4 hr of eclosion and aged in groups of three to five for 4 days. In a drop of 1× PBS on a charged slide (ProbeOn Plus microscope slides, FisherBiotech), reproductive tracts were dissected from these males, and the seminal vesicles were isolated. The seminal vesicles' walls were gently ruptured with dissecting forceps, and sperm were allowed to disperse for 10 min or until the 1× PBS had nearly evaporated. Sperm nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) per Sakaluk and O'Day (1984). DAPI-stained sperm were visualized using an Olympus BX50 epifluorescence microscope. The images were collected with a Pentamax CCD camera and displayed using MetaMorph software (Universal Imaging Corp.). Printouts from the computer-generated images of each slide were used to count all the sperm nuclei present in each seminal vesicle. As shown in Figure 1a, individual sperm nuclei are easily identifiable and countable.

Comparing sperm transfer: Given that a large number of sperm are transferred in a "viscous, tangled mass" (Yanders 1963) and that sperm immediately begin to disperse to the various sperm storage organs (Lefevre and Moore 1967), visual counting of sperm transferred to the female would be inaccurate and likely to result in a severe underestimate. Accordingly, we developed a molecular assay to quantify the total number of sperm transferred to females during mating (Figure 1). To determine the amount of sperm transferred, we used quantitative competitive PCR (reviewed in Zimmermann and Mannhalter 1996), a method that allows quantitation through the inclusion of a template-specific internal standard molecule.

Sperm transferred from DTA-D and control males can be specifically amplified from the genital tracts of their wild-type mates. Since these males are transgenic and the females to which they have been mated are not, sperm DNA is distinguishable from the large background of female DNA by its inclusion of transgene sequences. We chose as the template (Figure 1d) for amplification the sequence spanning the junction between

the *hsp70* promoter and the *white* reporter gene. These sequences are not juxtaposed in the wild-type genome, only in the transformation vector (Klemenz *et al.* 1987) used to generate the transgenic males. With an upstream primer to the *hsp70* promoter (5′AAGTAACCAGCAACCAAGTA-3′) and a downstream primer to the 5′ end of the *white* gene (5′-GATGTTGCAATCGCAGTTCCT-3′), we amplified a 433-bp product from transgenic flies. Under the same PCR conditions, no product was amplified from wild-type Oregon-R flies (Figure 1d).

To quantitatively compare the PCR-derived sperm signals, we included in each PCR reaction a competitor molecule that was specifically designed for the sperm target sequence (Figure 1d). The similarity in size and base composition between the target and competitor sequences allows for a more precise comparison because both will compete for the same primers and be amplified with similar kinetics (Diviacco *et al.* 1992; Zimmermann and Mannhalter 1996). The amount of starting template can be deduced by comparing the PCR signals of the template to that of the competitor. We constructed the competitor molecule by inserting 20 bp (5′-ACCT GCAGGGATCCGTCGAC-3′) into the middle of the *hsp70-white* junction template following the protocol of Diviacco *et al.* (1992). The competitor was cloned into the pTA plasmid (Invitrogen, Carlsbad, CA).

To quantify the amount of sperm transferred, we mated 3-day-old DTA-D or control males to 3-day-old Oregon-R virgin females. At the end of mating, genital tracts from the females were dissected in 1× PBS. The ovaries were removed, and the uteri were transferred to 0.7-ml microfuge tubes. Preparation of the tissues for PCR followed the protocol of Gloor *et al.* (1993), with minor modifications (Li *et al.* 1988). Groups of five uteri were homogenized in 40 μl squishing buffer (10 mm Tris-Cl, pH 8.2, 1 mm EDTA, 25 mm NaCl, 20 mm DTT, 0.02% SDS, and freshly added 200 μg/ml proteinase K). These homogenates were incubated for 1.5 hr at 37° and then 1–2 min at 95°. For each PCR reaction, we used 5 μl of homogenate (equivalent to 0.625 male ejaculate). PCR reaction conditions were as follows: 0.01% gelatin; 0.2 mm dNTP; 0.2 μm upstream primer; 0.2 μm downstream primer; 1.5 mm $MgCl_2$; 0.5 units Taq polymerase; and 4.6×10^{-5}, 4.6×10^{-6}, or 4.6×10^{-7} ng of competitor template. These amounts of competitor template are equivalent to 9615, 962, and 96 molecules, respectively. The amount of competitor to add was determined empirically by testing a dilution series of competitor molecule. We chose to use this series of three amounts because it defined the range, with an upper limit at ~15,400 sperm (9615 molecules/0.625 equivalent male ejaculate in the reaction) and a lower limit at ~154 sperm. When equal numbers of molecules of sperm and competitor are present, the equivalence point occurs, resulting in signals of equal intensity. The PCR cycle profile was 94° 5 min (94° 1 min, 50° 1 min, 72° 1 min) for 50 cycles and 72° 10 min. The PCR reactions were analyzed on 5% polyacrylamide gels.

On the basis of its amplification pattern in the PCR reactions, each sample was placed into one of five categories. Category I is defined by samples showing equal amplification of sperm DNA and 9615 molecules of competitor. Category II is defined by samples whose sperm signal was less intense than that of 9615 molecules but more intense than that of 962 molecules of competitor. Category III is defined by samples showing equal amplification of sperm DNA and 962 molecules of competitor. Category IV is defined by samples whose sperm signal was less intense than that of 962 molecules but more intense than that of 96 molecules of competitor. Category V is defined by samples showing equal amplification of sperm DNA and 96 molecules of competitor. No sample had a sperm signal of greater intensity than 9615 competitor molecules or of lesser intensity than 96 competitor molecules.

Comparing sperm storage: To determine the number of sperm stored by females, we directly counted sperm found in the seminal receptacle and spermathecae of the mated females. Adult flies were collected on ice within 4 hr of eclosion. Females and males were aged in groups of three to five individuals in separate vials containing yeasted medium. When the flies were 3–4 days old, DTA-D or control males were mated to Oregon-R virgin females. At the end of mating, females were moved to fresh vials and held individually for 0.5, 1, 3, or 6 hr, at which point their genital tracts were dissected out in 60% acetic acid. The ovaries were removed. Using fine forceps, the seminal receptacle was uncoiled, but left attached to the uterus, and the spermathecae were ruptured open by pinching between the tips of the forceps. The whole specimen was stained with 2% orcein in 60% acetic acid to label the nuclei (Lefevre and Jonsson 1962; Gilbert 1981). The samples were coded, and sperm in the seminal receptacle and spermathecae were counted at least two times for each sample. The repeat accuracy was to within 10%.

Data analysis: Data were analyzed by one-way ANOVA followed by Fisher's protected least significant difference on Statview version 4.1 (Abacus Concepts).

RESULTS

Males deficient in accessory gland secretions do not differ significantly from control males in sperm production: DTA-D males produce sperm (Kalb *et al.* 1993), but are poorly fertile (U. Tram and M. F. Wolfner, unpublished data). Their poor fertility could be due to insufficient sperm production by these males, to the inability of males to transfer sperm in the near absence of Acps, or to the inability of females to store or use sperm in the near absence of Acps. To assess sperm production in DTA-D males relative to control males, we counted and compared the number of sperm found in their seminal vesicles (Figure 1), where mature sperm are stored awaiting transfer to females during mating. The mean number (± SE) of sperm found in the seminal vesicles of DTA-D males (2014 ± 130, $n = 4$) was not significantly different from that of control males (1952 ± 52, $n = 4$) ($P = 0.674$). Since DTA-D males produce normal numbers of sperm, the lower number of sperm that are ultimately seen in storage (Kalb *et al.* 1993) must reflect a requirement for Acps in sperm transfer, storage, or both. Hence, we examined each of these phenomena quantitatively in DTA-D males.

Males deficient in accessory gland secretions transfer sperm with more variability and lower efficiency: Using competitive quantitative PCR, we measured and compared the relative amount of sperm transferred by DTA-D and control males. The quantity of sperm transferred was deduced by comparing the intensity of the sperm-derived PCR signal to that of the competitor. We then compared the amount of sperm transferred by DTA-D males relative to control males.

From its amplification pattern in the three competitive PCR reactions, each sample was placed into one of five categories (see materials and methods; Figure 2). The majority (14/21) of control samples showed

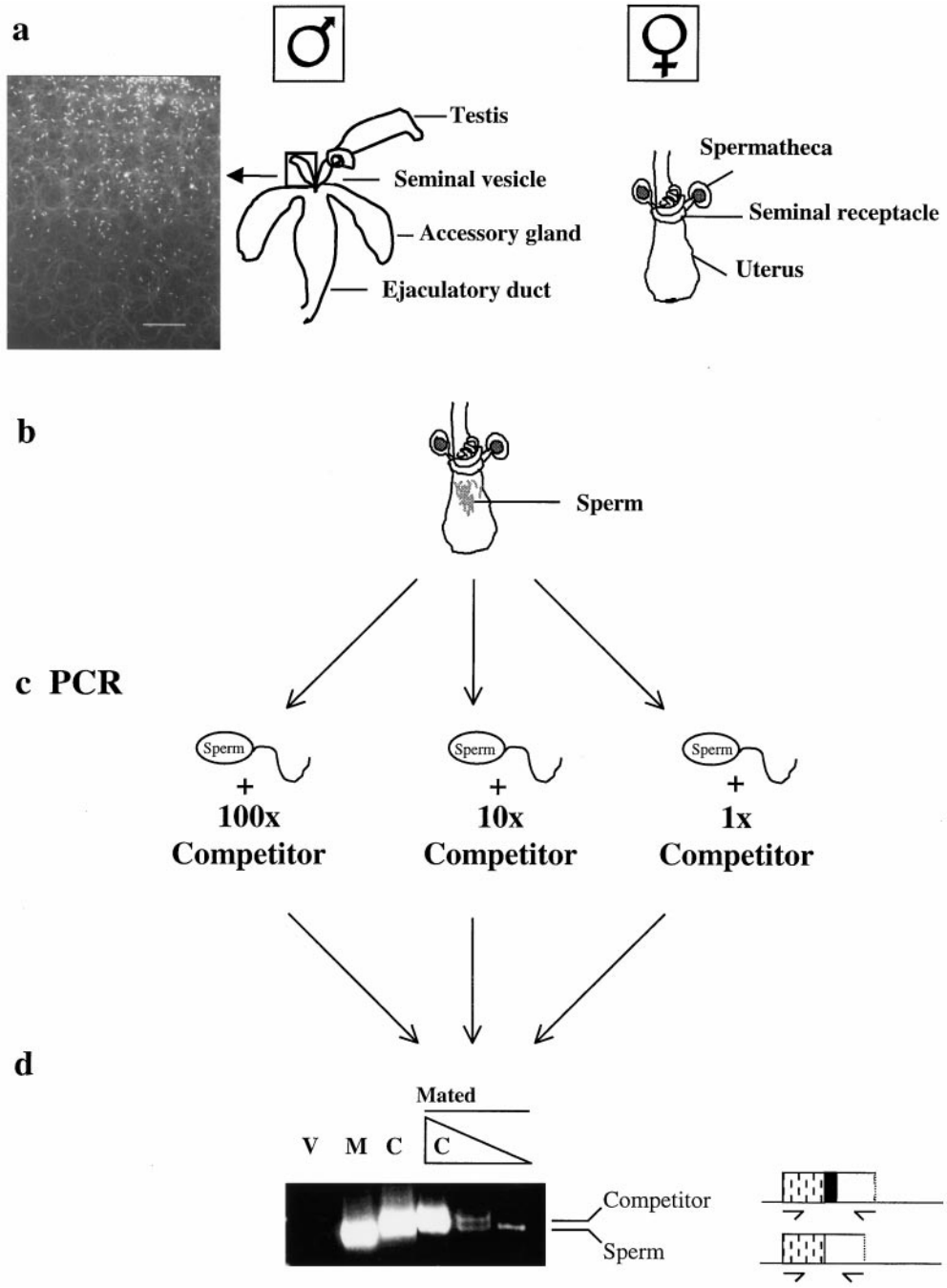

Figure 1.—Schematic of the competitive quantitative PCR protocol used to determine the amount of sperm transferred during mating. (a) This drawing of the male reproductive tract is modeled after that in Chen (1984). The paired testes produce sperm (left testis has been omitted for clarity), the paired seminal vesicles house mature sperm that will be transferred to females during mating, the paired accessory glands synthesize and secrete Acps, and the ejaculatory duct contributes additional seminal fluid proteins. (Inset) Sperm from seminal vesicles can be visualized easily after being stained with the fluorescent dye DAPI. This image represents one sector of a preparation from a seminal vesicle. The bar represents 100 μm. The drawing of the female reproductive tract is modeled after the drawing by Patterson and Stone (1952), as reprinted in Fowler (1973). The coiled seminal receptacle holds the majority of stored sperm. The pair of spermathecae store sperm for longer term usage. (b) Sperm are deposited in the uterus during mating. The quantity of sperm transferred can be determined by performing competitive quantitative PCR on genital tracts dissected from females immediately after mating. (c) For each sample, equal aliquots of the genital tract homogenate are used to set up three PCR reactions, each containing differing amounts of competitor molecules. (d) PCR reactions were then visualized on 5% polyacrylamide gels. A gel from a typical experiment is shown. The primers (arrows beneath the boxes representing the templates) specifically amplify a 433-bp product consisting of the 3′ end of *hsp70* promoter (stippled box) and the 5′ end of the *white* gene (open box) from transgenic males and their sperm (lane M) and no product from virgin Oregon-R females (lane V). These primers also amplify a 453-bp molecule (lane C) from a competitor plasmid containing the same 433-bp sequence with a 20-bp insertion (black box). In competitive PCR reactions ("Mated" lanes), the intensity of the signal derived from sperm DNA increases as the amount of competitor molecule decreases. The amount of sperm transferred can be deduced by determining the equivalence point, the reaction in which the sperm-derived signal is of equal intensity as the competitor-derived signal.

sperm transfers of >1540 sperm, in agreement with previous reports of the number of sperm transferred during mating (Kaufman and Demerec 1942; Gilbert 1981). Of the remaining 7 control samples, 4 showed sperm transfers equal to 1540 sperm, and 3 showed transfers between 154 and 1540 sperm. In contrast to this peak in distribution for control samples at 1540–15,400 sperm, DTA-D samples were more equally distributed among the five categories, with comparable numbers of samples showing sperm transfers >1540 sperm

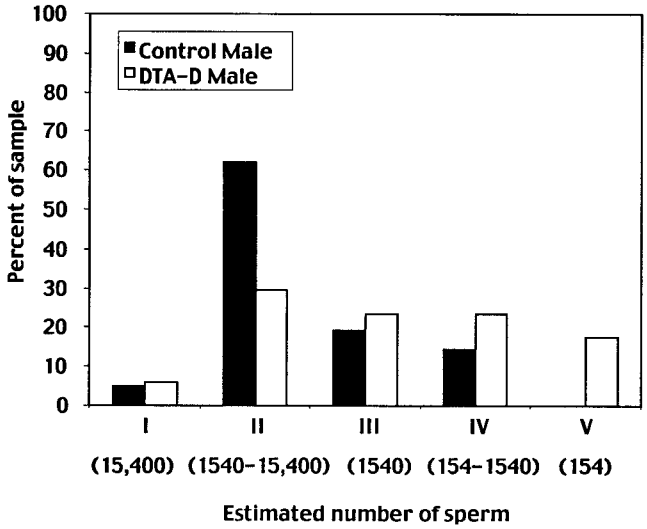

Figure 2.—Histogram of the quantity of sperm transferred by control and DTA-D males. Each sample was placed into one of five categories on the basis of its amplification pattern in the three competitive PCR reactions (see materials and methods). Fisher's exact test indicates that the amount of sperm transferred by DTA-D males is marginally significantly different from that of control males ($P = 0.0544$).

(6), transfers of ~1540 sperm (4), and transfers between 154 and 1540 sperm (7). These observations indicate that accessory gland main cell secretions are not required for the transfer of sperm during mating. Since DTA-D males transferred more variable numbers of sperm than control males, however, accessory gland main cell secretions might be important for the efficient transfer of large numbers of sperm.

To determine if this difference between DTA-D and control males is statistically significant, we performed a Fisher's exact test. We evaluated the statistical significance as a one-tailed test because the very low fertility of DTA-D males implied that they would either decrease or not affect (but not increase) the number of sperm transferred. For this analysis, we grouped categories together to form a "normal-to-high" transfer group and a "low" transfer group. Since *D. melanogaster* males can transfer several thousand sperm (3215–3877, Kaufman and Demerec 1942; 4690, Gilbert 1981) during mating, categories I and II were grouped together to form the normal-to-high (>1540) sperm transfer group, and categories III, IV, and V were grouped to form the low (≤1540) sperm transfer group. Fisher's exact test has relatively low statistical power to detect significant differences, but the more powerful tests require larger sample sizes and direct counts of sperm numbers. However, the marginally significant result in our analysis ($P = 0.0544$) and the clear difference between DTA-D and control males in the distribution of sperm transferred (Figure 2) suggest that while accessory gland secretions are not absolutely required for the transfer of sperm, they do play a role in the efficiency of sperm transfer.

Females mated to accessory-gland-deficient males store fewer sperm than females mated to control males: Kalb *et al.* (1993) reported that at 6 hr postmating, females mated to DTA-D males stored fewer sperm in their seminal receptacles than females mated to control males. Our analysis demonstrates that the lower amount of sperm in storage is not exclusively caused by receiving too few sperm during mating. Hence, Acps are required for some aspect of sperm storage. Acps may be required to get sperm into the sperm storage organs and/or into storage rapidly. Alternatively, Acps may be required for retaining sperm in storage rather than for getting sperm into storage. To define which aspect of sperm storage requires Acps, we compared the number of sperm found in the seminal receptacle and the spermathecae of females mated to DTA-D or to control males at various times, from 0.5 to 6 hr, postmating (Table 1). We did not examine timepoints later than 6 hr because females begin to lay eggs by 3–6 hr after mating (Gilbert 1981; Y. Heifetz and M. F. Wolfner, unpublished results). The movement of eggs through the genital tract will push out all or most sperm not stored by this time.

At each timepoint examined, females mated to control males stored significantly more sperm in both their seminal receptacle and spermathecae than females mated to DTA-D males (Table 1). For example, at 0.5 hr postmating, females mated to control males stored 501 ± 48 sperm in the seminal receptacle and a total of 128 ± 22 in both spermathecae, while females mated to DTA-D males stored only 7 ± 4 and 2 ± 1 sperm, respectively. The significant difference in sperm storage between control-mated and DTA-D-mated females persisted over the time course examined. The number of sperm stored by control-mated females peaked at 1 hr postmating, with 627 ± 34 sperm in the seminal receptacle and a total of 338 ± 41 sperm in both spermathecae. Females mated to DTA-D males, in contrast, had peak sperm storage at 6 hr postmating, storing 65 ± 25 sperm in the seminal receptacle and 10 ± 5 in the spermathecae. When Acps are transferred together with sperm, sperm storage occurs rapidly, being completed by 1 hr after the end of mating. In the absence of Acps, very few sperm get stored. Our results indicate that Acps are required for sperm to get stored, and they do not support a model in which sperm enter the sperm storage organs by "default," requiring Acps only to retain them in storage.

DISCUSSION

A male's mating success is ultimately measured by the number of progeny produced from his sperm. To gain a better understanding of how males maximize the possibility that their sperm will be used, we investigated the role of seminal fluid proteins produced by the male accessory gland main cells in mediating sperm transfer to and sperm storage in female *D. melanogaster*. Using

TABLE 1

Comparison of sperm storage in females that were mated to control or DTA-D males

	0.5 hr		1 hr		3 hr		6 hr	
No. of sperm	Control	DTA-D	Control	DTA-D	Control	DTA-D	Control	DTA-D
In seminal receptacle								
0–100	1[a]	22		15		21		12
101–200	1			1	2	1		2
201–300	1						1	3
301–400	1		1		1	1	1	
401–500	3		3		3		4	
501–600	4		3		7		6	
601–700	3		5		6		4	
701–800	3		4					
801–900			2				1	
901–1000							1	
Mean[b]	501 ± 48	7 ± 4	627 ± 34	27 ± 12	529 ± 35	33 ± 15	550 ± 35	65 ± 25
In spermathecae								
0–50	3[a]	22		16	1	23	1	15
51–100	5		1		1		1	1
101–150	3		1				2	
151–200	2		2		2		3	
201–250	2				3		1	
251–300	1		3		3		2	
301–350	1		2		3		1	
351–400			1		3			
401–450			1				1	
>451			4				4	
Mean[b]	128 ± 22	2 ± 1	338 ± 41	3 ± 2	249 ± 26	2 ± 1	267 ± 39	10 ± 5

[a] Number of females having this number of sperm in the indicated storage organ.

[b] Mean number of sperm stored ± SEM. Females mated to DTA-D males store significantly fewer sperm than females mated to control males at all timepoints examined (all $P < 0.0001$).

genetically altered males that produce almost no accessory gland main cell secretions, we demonstrated that males are capable of transferring sperm in the near absence of accessory gland secretions, but that their mates store very few of those sperm. Thus, accessory gland proteins are crucial for mediating sperm storage.

We applied a novel approach to quantifying the number of sperm transferred during mating. In the past, the number of sperm transferred had been determined by direct counts of sperm in fixed and stained female genital tracts (Kaufman and Demerec 1942; Gilbert 1981). This process is labor intensive, time consuming, and can be inaccurate. Adopting competitive quantitative PCR, a molecular method that is routinely used to quantify the number of mRNA molecules and viruses (Lee *et al.* 1996; Vener *et al.* 1996; Borson *et al.* 1998), we were able to quantify the amount of sperm that was transferred during mating. With this method, we found that the majority of control males transfer between 1540 and 15,400 sperm. These numbers are in accordance with numbers reported previously by Kaufman and Demerec (1942), 3215–3877 sperm transferred by 6-day-old males, and by Gilbert (1981), observed maximum of 4690 sperm transferred by 3- to 5-day-old males. Thus, this molecular method can be applied to other assays where quantitation is required but direct counting is difficult or impossible.

We observed that accessory gland main cell-deficient males are able to transfer sperm during mating. This indicates that Acps are not absolutely required for sperm transfer, lending support to the conclusion of Hihara (1981). That DTA-D males do not transfer more sperm than control males also suggests that the amount of sperm drawn from the seminal vesicles is not dependent on the volume of accessory gland secretions available for producing seminal fluid. Thus, the transfer of sperm and other seminal fluid components from the male reproductive tract may be a purely mechanical mechanism whereby each component is transferred independently of one another. Though no single study has examined all secretions simultaneously, available data about individual components of the seminal fluid suggest that secretions from each male reproductive tissue are transferred to the female in succession rather than in tandem. Acps (Lung and Wolfner 1999) and esterase-6 (Richmond and Senior 1981), an enzyme produced by the ejaculatory duct, are detected in the female genital tract within 3 min after the start of mating. By 5–6 min, *cis*-vaccenyl acetate, a lipid from the ejaculatory bulb, is present in the female tract (Scott and Rich-

mond 1987). By 5–7 min, a mating plug has formed in the uterus (Bairati and Perotti 1970). The mating plug is hypothesized to act as a scaffold that aids sperm to move up the female genital tract (Bairati 1968). Finally, sperm are transferred to the uterus at 7–10 min and are found in the sperm storage organs at 12 min (Bairati and Perotti 1970).

Though sperm can be transferred in the near absence of Acps, the efficiency of their transfer is compromised. While control males generally transferred >1540 sperm, DTA-D males transfer anywhere from 154 to >1540. Accessory gland main cell secretions may thus be important for mediating efficient sperm transfer. These secretions may function as lubricants or fibrous tracks that guide sperm, preventing them from adhering to parts of the male genital tracts that would hinder their passage into the female.

We have shown here that successful sperm transfer to females does not automatically lead to storage of those sperm. Though the majority of females mated to DTA-D males receive more sperm than they are capable of storing, they store fewer sperm than females mated to control males. While females mated to control males are capable of storing >1000 sperm by 1 hr postmating (627 ± 34 sperm in the seminal receptacle and 338 ± 41 sperm in both spermathecae), females mated to DTA-D males store only 100 sperm by 6 hr postmating (65 ± 25 sperm in the seminal receptacle and 10 ± 5 sperm in both spermathecae). The observed lag in time required for maximum sperm storage indicates that accessory gland main cell secretions are required for the rapid and efficient storage of sperm by females. Females receiving the complete complement of seminal fluid components finish storing sperm in their seminal receptacle and spermathecae within the first hour after the end of mating. Gilbert (1981) also observed that maximum storage in the seminal receptacle occurs by 0.9 hr after mating, but reported peak storage in the spermathecae at 5 hr after mating. The difference seen in the results on the spermathecae in the two studies may be caused by differences in the strains used in the studies. Sperm storage needs to occur rapidly, since the first egg to be ovulated and move down the genital tract to be fertilized and laid will push out sperm that have not moved out of the uterus and into the sperm storage organs. In Oregon-R females mated to Oregon-R males, an egg can be seen in the uterus by 1.5 hr after the end of mating (Y. Heifetz and M. F. Wolfner, unpublished results). When females are mated to mutant males lacking Acp26Aa, an accessory gland protein with a region of similarity to an egg-laying hormone of the sea hare *Aplysia californica* (Herndon and Wolfner 1995), this rapid induction of ovulation is not observed (Y. Heifetz, O. Lung, E. Frongillo and M. F. Wolfner, unpublished results). Thus, females mated to DTA-D males (which express Acp26Aa at 1% the level of control males, Kalb *et al.* 1993) may be able to continue to store sperm for 6 hr after the end of mating because sperm have not been pushed out of the uterus by ovulated eggs.

Accessory gland secretions may stimulate the female genital tract to contract. These contractions could transport sperm into the sperm storage organs. Accessory gland secretions may also stimulate sperm to migrate actively into the sperm storage organs. That females are able to store sperm at all in the near absence of Acps suggests that other seminal fluid components could be involved in mediating sperm storage. One candidate is accessory gland secondary cell filaments (Bairati 1966; Perotti 1971). The secondary cells comprise 4% of the accessory glands, and they are unaffected in DTA-D males (Kalb *et al.* 1993). Bairati and Perotti observed fibers that resemble secondary cell fibers in the lumen of the accessory gland and in the sperm storage organs of the mated female. They hypothesized that the fibers are transferred to the female during mating and enter the sperm storage organs. They hypothesized that the fibers play a mechanical role by providing a scaffold along which sperm travel or acting as "contractile, motile" elements that help sperm motility.

While the focus has been on identifying male components required for sperm storage in females, it has been recently shown that females exert some influence on the fate of the sperm in their genital tract. Clark and Begun (1998) demonstrated that sperm competition is affected by the genotype of the female. *D. melanogaster* females, as well as females of other species examined, show a tendency to use sperm from the last male to mate with them. Using three series of female lines, each homozygous for one of the three autosomes, Clark and Begun compared the proportion of total progeny that are produced from the last male to copulate. They demonstrated that the variability in the pattern of sperm use can be correlated to the genotype of the female. Female genotype is hypothesized to manifest its effect on sperm storage and usage through differential responses to male-provided signals (Clark and Begun 1998), which we demonstrate here are Acps.

Though sperm storage is an important reproductive strategy used by females in many animals ranging from insects to mammals (reviewed in Neubaum and Wolfner 1999a), its molecular mechanism is not known. Our observations identify Acps as important molecules that are necessary for females to store sperm. Identification of the male accessory gland as the source of proteins essential for sperm storage by *D. melanogaster* females focuses the search for important molecules for this phenomenon on the products of this male tissue. The following paper describes a mutational analysis that identifies one accessory gland protein, Acp36DE, as essential for sperm storage, providing a molecular entry point into this important process (Neubaum and Wolfner 1999b).

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