Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 10114–10118, October 1995
Genetics

A *Drosophila* seminal fluid protein, Acp26Aa, stimulates egg laying in females for 1 day after mating

(accessory gland/egg-laying hormone/reproduction/sperm competition/mating behavior)

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Communicated by M. M. Green, University of California, Davis, CA, July 25, 1995 (received for review June 5, 1995)

ABSTRACT **Mating triggers behavioral and physiological changes in the *Drosophila melanogaster* female, including an elevation of egg laying. Seminal fluid molecules from the male accessory gland are responsible for initial behavioral changes, but persistence of these changes requires stored sperm. Using genetic analysis, we have identified a seminal fluid protein that is responsible for an initial elevation of egg laying. This molecule, Acp26Aa, has structural features of a prohormone and contains a region with amino acid similarity to the egg-laying hormone of *Aplysia*. Acp26Aa is transferred to the female during mating, where it undergoes processing. Here we report the generation and analysis of mutants, including a null, in *Acp26Aa*. Females mated to male flies that lack Acp26Aa lay fewer eggs than do mates of normal males. This effect is apparent only on the first day after mating. The null mutation has no other detectable physiological or behavioral effects on the male or the mated female.**

After mating, *Drosophila melanogaster* females undergo changes in their behavior and physiology. These changes persist for several days and include a stimulation of egg laying, a decrease in receptivity to further mating, and storage and utilization of sperm. In addition, mated females have a shorter life-span than unmated females (1, 2). *D. melanogaster* provides a genetic system to identify and examine the molecules that cause the changes in mated females. In this paper, we report that a molecule with sequence similarity to a hormone that stimulates egg laying in the mollusc *Aplysia* (3, 4) is involved in stimulating egg laying in *D. melanogaster*.

Elevation of egg laying in *Drosophila* has two phases, a short-term phase that lasts 1 day and a long-term phase that lasts 7–9 days. Initially, egg laying is triggered by the transfer of seminal fluid to the female. This effect lasts ≈1 day (5). The long-term phase of increased egg laying requires the storage of sperm (5, 6). The seminal fluid components that stimulate egg laying derive from the accessory gland, a secretory tissue of the male reproductive tract. Transplantation of whole accessory glands into the abdomens of unmated females stimulated egg laying and decreased receptivity to mating to levels similar to those seen in mated females (7, 8). Mates of male flies producing accessory gland secretions, but no sperm, show an increase in egg laying for 1 day only; mates of males lacking both accessory gland secretions and sperm show no stimulation (5).

To identify products in accessory gland secretions, Chen *et al.* (9) assayed fractionated accessory gland extracts by injection into females. This process led to purification of a peptide (sex peptide) that stimulates egg laying for 1 day when injected in estimated physiological amounts into unmated females. Sex peptide can also decrease receptivity to mating for 1 day when introduced into unmated female flies (9). However, in at least one other *Drosophila* species in which a sex peptide exists, other molecules can also cause increased egg laying and decreased receptivity (10). The discovery of Accessory gland protein (Acp) 26Aa, a prohormone-like protein with sequence similarity to the egg-laying hormone (ELH) of *Aplysia californica* (3, 4), suggested that multiple factors could be involved in egg laying in *D. melanogaster*. We therefore used, and report here, a genetic approach to isolate flies mutant in their production of Acp26Aa.

Acp26Aa is a 264-amino acid protein with an 18-amino acid signal sequence targeting it for secretion. While the predicted molecular mass of the protein (after removal of the signal sequence) is 28 kDa, its apparent molecular mass is between 9 and 13 kDa higher due to N-linked glycosylation (3, 11). During mating, Acp26Aa is transferred to the female genital tract and rapidly enters the hemolymph, where it persists for several hours (11). In the genital tract, Acp26Aa undergoes very specific processing similar to what is seen in the liberation of bioactive peptides (11, 12). While these features of Acp26Aa are consistent with a possible role in egg laying, another study suggested that Acp26Aa might be involved in a different physiological change in the mated female. Clark *et al.* (13) observed that natural polymorphisms at the *Acp26Aa* locus correlated with the ability of a male's sperm to defend against displacement by a second male's sperm. Thus, Acp26Aa was suggested to play a role in sperm competition.

Here we report the isolation of three mutants in Acp26Aa by using a screen to identify flies that lack the Acp26Aa protein. Phenotypic analysis of these mutants showed that females mated to males that lack Acp26Aa do not lay as many eggs on the first day as females mated to normal males. Therefore, Acp26Aa contributes to the initial (first day post-mating) stimulation of egg laying in the mated female.

MATERIALS AND METHODS

Isolation of *Acp26Aa* Mutants. Three-day-old OregonR males (+/+) were treated with 25 mM ethyl methanesulfonate according to Lewis and Bacher (14). Treated males were mated en masse to *CyO/Gla* virgin females. Individual male progeny carrying the mutagenized chromosome (*) over *Gla* were mated to three *Df(2L)PM101/SM1* virgin females to produce lines [*Df(2L)PM101* deletes 25E1-2–26A2-5]. F_2 males [*/*Df(2L)PM101*] were screened for Acp26Aa production on dot blots by minor modifications of the procedures of Van Vactor *et al.* (15) and by using a horseradish peroxidase-conjugated secondary antibody (Amersham). Lines were established from males having decreased amounts of Acp26Aa by crossing mutant males to *Df(2L)PM101/SM1* females. Lines were maintained by repeating this cross with */*SM1* males each generation so that the mutagenized chromosome is kept over the balancer to avoid selection pressures.

Measurement of Male-Specific Protein Production in *Acp26Aa* Mutant Males. Proteins from male genital tracts were

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Abbreviation: ELH, egg-laying hormone.

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detected on Western blots as described by Monsma and Wolfner (3) with minor modifications. Affinity-purified antibodies were used against Acp26Aa and Acp26Ab (3), Acp36DE (M. J. Bertram and M.F.W., unpublished work), and Esterase 6 (ref. 16; kindly provided by R. C. Richmond).

Nucleic Acids. Quantitation of poly(A)$^+$ *Acp26Aa* RNA from 3-day-old virgin */Df(2L)PM101* or +/*Df(2L)PM101* males was done with Northern blots according to the procedures of DiBenedetto *et al.* (17). Signals from probing with an *Acp26Aa* probe were quantified with a Betascope or by densitometry and were normalized to those of a β1-tubulin probe (18).

To identify the mutant lesions, DNA from the mutant animals was PCR amplified (19) using two 5′ primers complementary to exonic sequences −79 to −60 and +132 to +151 and a 3′ primer complementary to +830 to +849 according to the sequence reported by Monsma and Wolfner (3) as modified by Aguadé *et al.* (20). Amplified DNAs were sequenced by the dideoxynucleotide chain-termination method (21).

Fertility, Receptivity, Egg Laying, and Sperm Displacement in the Mates of *Acp26Aa* Mutant Males. To measure fertility, egg laying, receptivity, and sperm displacement of mates of *Acp26Aa* males relative to controls, females mated to */Df(2L)PM101* males were compared with females mated to +/*Df(2L)PM101* males generated in a cross of flies from the parental OregonR stock to *Df(2L)PM101/SM1*. The female and male flies were collected within a few hours of eclosion, stored separately in groups of 10 on fresh yeast glucose/acid mix food (22), and aged 3–5 days at 23.5 ± 0.5°C. To measure fertility, we counted total progeny from single females during a 10-day period after a single mating to a mutant or a control male. Similarly, egg production by individual females after a single mating was scored by counting the number of eggs laid in successive 24-hr periods; females were transferred to new vials each day for the length of the experiment. To measure receptivity 24 hr postmating, we compared the mates of mutant or control males for their willingness to remate by using assays described by Kalb *et al.* (5). Sperm competition experiments were done to test for the ability of a first male's sperm to resist displacement by a second male's sperm ("defense") as described by Clark *et al.* (13). Four-day-old *cn bw* females were mated first to 4-day-old mutant or control virgin males and after 2 days were remated to 4-day-old *cn bw* virgin males. Progeny of each genotype were counted. Statistical tests on all data were performed with the computer program StatView, version 4.02.

RESULTS

Isolation of Three Lines Mutant in Production of Acp26Aa. To be able to test the function of Acp26Aa, we needed to isolate and analyze mutants that lacked it. Since flies that make low levels of accessory gland secretions are fertile (5), it is unlikely that mutation in any single accessory gland gene (such as *Acp26Aa*) would result in complete sterility. We therefore isolated mutants in *Acp26Aa* by methods that did not presuppose a function for the protein (15), allowing us to isolate fertile or sterile mutants.

Among 2823 mutagenized lines of flies screened, we isolated three independent lines in which Acp26Aa was not detected when individual male flies were homogenized, and their proteins were dot blotted and probed with antibodies. On Western blots of accessory gland proteins from males from line 1, we do not detect any Acp26Aa protein (Fig. 1*A*, lane 2). On Western blots of accessory gland proteins from lines two and three, however, we detected a 21- and 25-kDa doublet with Acp26Aa antibodies (lane 3). This doublet is 5–10 times less abundant than Acp26Aa in wild-type flies and is 16 kDa smaller than wild-type Acp26Aa, which also runs as a doublet at 37 and 41 kDa due to differential glycosylation (3) (lane 1).

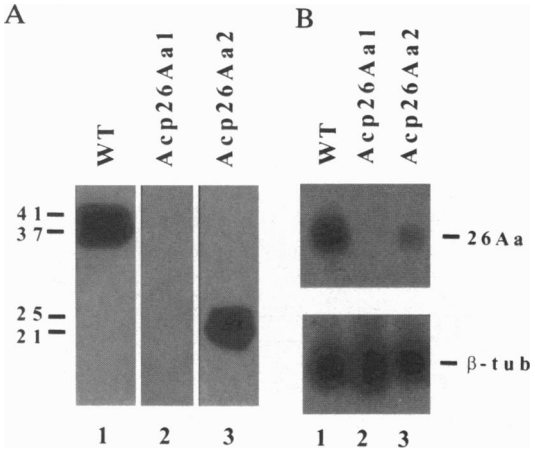

FIG. 1. (*A*) Acp26Aa in *Acp26Aa* mutants. Lanes: 1, extracts of two accessory gland pairs from 3-day-old OreR virgin males (WT, wild type); 2 and 3, extracts from 13 *Acp26Aa1* (lane 2) and 20 *Acp26Aa2* (lane 3) accessory gland pairs from 3-day-old virgin males probed with anti-Acp26Aa as described (3). Lane 3 shows protein from line 2 flies. The same was observed from line 3 flies (data not shown). Lanes 1 and 3 are from the same blot; lane 2 is from a different blot, run and exposed under the same conditions with a standard that appears exactly like lane 1. (*B*) RNA blot analysis of *Acp26Aa* mutants. Lanes 1–3 contain ≈10 μg of poly(A)$^+$ RNA isolated from control, *Acp26Aa1*, and *Acp26Aa2* male flies. (*Upper*) Probing of this blot with a full-length *Acp26Aa* cDNA clone (3). (*Lower*) Reprobing of the same blot for β1-tubulin RNA (18) as a loading control. The amount of *Acp26Aa* mRNA was normalized relative to the amount of β1-tubulin RNA. *Acp26Aa* mutant line 3 gave the same results as those shown here for line 2.

To narrow down the nature of the alteration of Acp26Aa in the mutant lines, we quantified the level and size of *Acp26Aa* mRNA in each mutant line (Fig. 1*B*). Males from line 1 contain little, if any, transcript of *Acp26Aa* (<5% of wild type, the detection limit). Males from lines 2 and 3 produce a normal sized *Acp26Aa* transcript at ≈30% the level of control flies.

Due to the genetics of the mutant isolation screen, the location of the mutations was most likely within the *Acp26Aa* gene. Therefore, we sequenced *Acp26Aa* genomic DNA for each of the mutant chromosomes to determine the molecular nature of the mutant lesions. The first mutant line contains a nonsense mutation at amino acid 43, replacing lysine with a stop (Fig. 2). This would result in truncation of Acp26Aa to a 25-amino acid peptide after signal sequence cleavage (23). The presence of the stop codon and the lack of detectable transcript suggest that the *Acp26Aa* mRNA might be destabilized in these lines by the arrest in translation (24). This allele of *Acp26Aa*, which appears to be a null allele, is designated *Acp26Aa1*.

Lines 2 and 3 contain identical nonsense mutations at amino acid 166. Designated *Acp26Aa2*, this allele results from a C to T transition, replacing a glutamine with a stop codon. The location of the stop codon predicts an *Acp26Aa*-encoded protein that is about two-thirds the size of the normal protein. This is consistent with the observed size of the mutant protein on Western blots. The predicted truncated protein still contains the region of sequence similarity to the *Aplysia* ELH (3). If this region contains the bioactive region of the protein, the truncated version could retain some activity. For this reason, we limited our phenotypic analysis to the *Acp26Aa1* mutant.

***Acp26Aa1* Males Appear Normal.** Male flies lacking Acp26Aa are of normal viability and appear morphologically normal externally and internally. Their accessory glands are filled with secretions and their testes contain large numbers of motile sperm (data not shown). At the molecular level, production of other accessory gland products [Acp26Ab (3) and Acp36DE (M. J. Bertram and M.F.W., unpublished data)] is

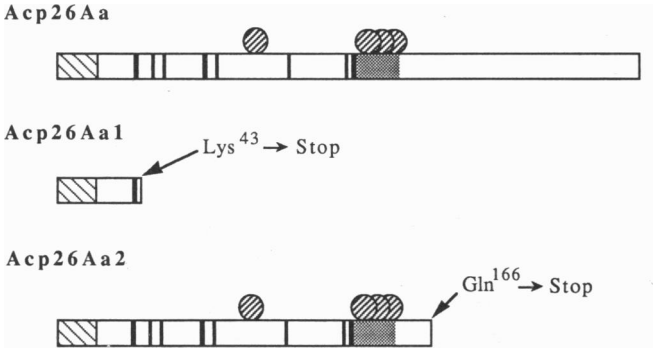

FIG. 2. Diagram of wild-type and mutant Acp26Aa proteins. Hatched boxes, predicted hydrophobic signal sequence; hatched circles, potential N-linked glycosylation sites (Asn-Xaa-Ser/Thr); vertical bars, sequences that resemble potential prohormone cleavage sites (the 2nd, 4th or 5th, and 7th or 8th are apparently used) (12). Shaded region represents region of similarity to the ELH of *A. californica* (3). In *Acp26Aa1*, the protein is predicted to be truncated as a result of a stop codon at amino acid 43 (AAA → TAA). The mutation arose in a stock polymorphic at two positions (no. 136 A/G and no. 214 C/G) previously described for *D. melanogaster* (20). The mutant chromosome carries G at both positions. In *Acp26Aa2*, the protein is predicted to be truncated at position 166 (CAG → TAG) as a result of a stop codon generated at this position.

unaffected, as is synthesis of Esterase 6, a product of the ejaculatory duct (16) (data not shown). That production of Acp26Ab is normal in these flies is of particular interest since its gene is very tightly linked to *Acp26Aa* with only 20 bp separating the 3′ end of *Acp26Aa* mRNA from the start of *Acp26Ab* mRNA (3). Thus, the mutations in Acp26Aa are gene specific and do not alter the production of other male products. Since Acp26Aa is passed to the female during mating, we can examine the effect of mutation in this gene by looking at processes in females known to be affected by the transfer of accessory gland products from the male.

Egg Laying Is Decreased in Mates of *Acp26Aa1* Males for 1 Day After Mating. To determine the effect of Acp26Aa on egg laying, we compared the number of eggs laid daily by mates of control and mutant males. Significant differences were seen in our first sets of experiments. To increase our sample size and to be sure the effects we observed were not due to fluctuations in medium or to the time of year, we repeated the experiment 15 times. There was variation between the experiments, but in 10 of the 15 experiments, females mated to *Acp26Aa* mutant males laid fewer eggs than females mated to control males for 1 day after mating; three of these experiments showed statistically significant differences. Of the other five experiments, four showed a trend in the opposite direction and in one females mated to mutant and control males laid equivalent mean numbers of eggs, but none of these differences were statistically significant.

To compare all the experiments while controlling for variations that might be present from experiment to experiment, we used a block design for ANOVA. Significant differences ($P = 0.0422$) were found between the numbers of eggs laid by mates of control and mutant males, but only for the 1st day after mating (Table 1). Mates of mutant males laid a mean of 3.47 fewer eggs than mates of the control males. With mates of control males laying a mean of ≈42 eggs on the 1st day, this difference results in an ≈8% decrease in egg laying on the 1st day when females mate with males that do not produce Acp26Aa. However, the next day (2 days after mating) we did not see significant differences in the number of eggs laid by mates of mutant or control males ($P = 0.4469$) (Table 1). Similarly, on day 3 (four experiments performed) and day 4 (three experiments performed), no statistically significant differences were seen between mates of the mutant or control males ($P = 0.3716$ and 0.5067, respectively). These experiments provide a particularly good control because they involve the same females that laid significantly different numbers of eggs on day 1. Thus, lack of Acp26Aa in males causes a small but statistically significant decrease in egg laying by their mates but only for 1 day after mating.

Fertility, Receptivity, and Sperm Competition in Mated Females Is Not Affected by Lack of Acp26Aa. Lack of Acp26Aa does not affect the fertility of the male. In a representative experiment, mates of 13 mutant males had a mean of 263 ± 45 progeny, while mates of 11 control males had a mean of 241 ± 43 progeny. These two numbers are not statistically different when compared by *t* test ($P = 0.085$).

Lack of transferred Acp26Aa does not affect the mated female's change in receptivity 24 hr after mating. In a representative experiment, 0/12 females previously mated to control males remated, 0/18 females previously mated to mutant males remated, and 15/16 virgin females mated.

Previous studies by Clark *et al.* (13) suggested that Acp26Aa might be involved in "defense," the ability of a first male's sperm to resist displacement by a second male's sperm. However, we found that absence of Acp26Aa does not affect the ability of the first male's sperm to resist displacement. Similar results were obtained by A. G. Clark (personal communication). Sperm from mutant or control males were compared in their ability to resist displacement by sperm of a second male. This comparison was performed by measuring the fraction of progeny sired by the first male and is designated as the statistic P1. First, 262 virgin females were mated to control males and then after 2 days they were mated to *cn bw* males. In parallel, 252 virgin females were mated to mutant males and then after 2 days they were mated to *cn bw* males. In the control mating, 32% ± 16% of progeny were derived from the first male (P1 = 0.3186), whereas 30% ± 18% (P1 = 0.3022) of progeny in mutant matings were derived from the first male. These values are not statistically different ($P = 0.1285$).

DISCUSSION

In both invertebrates and vertebrates, hormones act on different tissues and affect reproductive behavior. *D. melanogaster* females lay a large number of eggs after receiving accessory gland products from the male during mating. In this study, we used a genetic approach to generate mutants in an accessory gland protein, Acp26Aa, and demonstrated that it is

Table 1. ANOVA of numbers of eggs laid after mating

Day	Source of variation	DF	Sum of squares	Mean square	*F* value	*P* value
1	Male	1	1,641.657	1641.657	5.31	0.0422
	Male* Exp	14	4,480.108	320.007	2.30	0.0044
	Residual	665	92,609.271	139.262		
2	Male	1	46.101	46.101	0.581	0.4469
	Male* Exp	5	902.484	180.497	2.275	0.0490
	Residual	181	14,361.811	79.347		

DF, degrees of freedom.

involved in stimulation of egg laying. Mates of males that lack Acp26Aa do not lay as many eggs as mates of normal flies on the 1st day after mating. Consistent with a role in egg laying, Acp26Aa has a region of amino acid sequence similarity to the egg-laying hormone of *A. californica*. Acp26Aa has structural features of a prohormone and is transferred to the female genital tract, where it undergoes processing (3, 11). Acp26Aa also rapidly enters the hemolymph (11) from which it has access to the brain, thoracic ganglia, and corpora allata. These organs are implicated in egg laying behavior from experiments using gynandromorphs or flies that have had parts of their brain microcauterized (25–28). All of these characteristics suggested that Acp26Aa could act on the mated female as an egg-laying hormone.

That the effect of Acp26Aa on egg laying does not persist beyond 1 day is consistent with results of previous studies of accessory gland function. Even though mating elevates egg laying for 7–9 days, only the 1st day of this is due to seminal fluid components. This was demonstrated by Kalb *et al.* (5), who showed that virgin females (which lay very few if any eggs) receiving only seminal fluid without sperm (from males that produce no sperm) lay a mean of 26 ± 17 eggs for the 1st day after mating. Without the transfer of sperm, the females' egg-laying rate drops close to the level of virgin females after 1 day. Kalb *et al.* (5) showed that accessory gland secretions and sperm are the only genital secretions required for the full short-term peak in egg laying. Consistent with the short-term effects of accessory gland products, Acp26Aa is detectable for only 3 hr in the genital tract and 6 hr in the hemolymph after the end of mating (11).

Elimination of Acp26Aa has only a small effect on egg laying. The most likely explanation is that several independent factors play somewhat redundant roles in stimulating egg laying. Such a phenomenon was observed in *Drosophila suzukii*, which produces OSS (ovulation stimulating substance) (10) and sex peptide (34), each of which on its own can fully stimulate egg laying when injected into virgin females. *D. melanogaster* male accessory glands also contain sex peptide. Since sex peptide is likely to stimulate egg laying when passed to females during mating, its presence in the seminal fluid of Acp26Aa null males may mask the full effect of the lack of Acp26Aa in the mutant flies.

It is difficult to determine the relative contribution of sex peptide or Acp26Aa in the stimulation of egg laying since the assays used to determine their function are not compatible. No mutants in sex peptide exist to allow us to compare the phenotypes of single and double mutants lacking sex peptide, Acp26Aa, or both. Such comparisons could help determine the relative roles of these two molecules in the stimulation of egg laying and could reveal whether other redundant functions exist. Conversely, Acp26Aa produced in *Escherichia coli* had no effect on egg laying when injected into an unmated female (29). This could be due to improper modifications of Acp26Aa when produced by *E. coli*, improper targeting of the protein, or an inability of the protein to act alone.

It is possible Acp26Aa is required to optimize egg laying under environmental conditions, which we have not fully replicated in the laboratory. If present in a natural population, a mutation in *Acp26Aa* could have a much more dramatic effect on egg laying than we detected. Egg laying is very susceptible to variations in food conditions (30). Our experiments were spread out over the course of a number of months to control for effects of medium and different times of year. Despite variation between experiments presumably due to these conditions, the overall significance of the result remained consistent.

When sex peptide or OSS is introduced into unmated females, these flies show both an increase in egg laying and a decrease in receptivity (9, 10, 31). Synthetic fragments of sex peptide elicit both or neither of these effects, suggesting that the two response cascades are not separable at their initiation (32). Females mated to *Acp26Aa1* males did not show any differences in their response to courtship when compared to females mated to normal males. Our experiments suggest that receptivity does not depend on Acp26Aa and that its effect on egg laying is via a pathway different from receptivity. This could be due to Acp26Aa acting "downstream" of or independent of sex peptide. For example, by analogy to one of the functions of ELH in *Aplysia* (33), Acp26Aa might act directly on the musculature of the genital tract to stimulate contractions, resulting in rapid deposition of any matured eggs. However, we cannot rule out that transfer of sex peptide or another molecule that functions in receptivity could mask the possible involvement of Acp26Aa.

That the fertility of mates of the Acp26Aa mutant males is normal is not surprising. Since lack of Acp26Aa results in a decrease in egg laying for only 1 day after mating, and this effect is small, it does not affect the total number of progeny produced over a 10-day period. This is not to say, however, that in natural populations this decrease in the number of eggs laid on the 1st day would not have a large effect on the fitness of these flies. There could still be strong selection pressures against flies that do not produce a maximum number of progeny on the 1st day. Studies by Aguadé *et al.* (20) showed that the distribution of polymorphisms over the *Acp26Aa* region is inconsistent with the pattern of divergence between different species of *Drosophila*. These results suggest that selection in or near this locus has played a role in the evolutionary history of this gene.

Clark *et al.* (13) observed that polymorphisms at Acp26Aa in natural populations correlated with the ability of sperm to resist displacement by subsequent sperm. However, we observed no effect on sperm competition by an absence of Acp26Aa, using the same assay paradigm in which Clark *et al.* saw this correlation. If Acp26Aa participated in sperm competition, one would have expected that its absence would result in a decrease in the ability of these mutant males' sperm to resist displacement. It is possible that the data of Clark *et al.* (13) implicate a gene other than *Acp26Aa*. Another accessory gland protein, Acp26Ab, is encoded 20 bp downstream of *Acp26Aa*. This tightly linked gene, or another unidentified one in the region, could be responsible for the allele-dependent variation in sperm displacement seen by Clark *et al.* (13). Alternatively, it is possible that there is functional redundancy in the molecules that mediate sperm displacement. This could mask the effect of the loss of a single protein such as Acp26Aa.

Since we have identified a role for Acp26Aa in the mated female, a next step will be to identify targets for the protein in the female. With its presence in both the genital tract and the hemolymph, there are a variety of places Acp26Aa could target. Acp26Aa either could act from within the genital tract, or it could act as a hormone via the hemolymph and target the brain, nervous system, or endocrine organs, such as the corpora allata. In addition, an extension of the type of genetic analysis done here to other accessory gland proteins should bring us closer to understanding how molecules transferred to the female during mating change her behavior and physiology.

We are very grateful to A. G. Clark for helping with the statistical analysis of our data, for communicating unpublished results, and for commenting on the manuscript. We thank R. MacIntyre for assistance in designing the crosses and for comments on the manuscript. D. Begun provided technical advice for sequencing the mutant alleles. R. Richmond kindly provided antibodies to Esterase 6. G. Churchill, M. Ford, and M. Nachman helped in statistical analysis of the data. C. Aquadro, J. Kalb, K. Kemphues, S. Monsma, D. Neubaum, and M. Park provided helpful comments on the manuscript. This work was supported by sequential grants to M.F.W. from the National Science Foundation. For part of the work L.A.H. was supported by National Institutes of Health Training Grant T32-GM07617.

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