Acp36DE is required for uterine conformational changes in mated *Drosophila* females

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In a multitude of animals with internal fertilization, including insects and mammals, sperm are stored within a female's reproductive tract after mating. Defects in the process of sperm storage drastically reduce reproductive success. In *Drosophila* males, ''Acp'' seminal proteins alter female postmating physiology and behavior, and are necessary for several aspects of sperm storage. For example, Acps cause a series of conformational changes in the mated female's reproductive tract that occur during and immediately after mating. These conformational changes have been hypothesized to aid both in the movement of sperm within the female and in the subsequent storage of those sperm. We used RNAi to systematically knock down several Acps involved in sperm storage to determine whether they played a role in the mating-induced uterine conformational changes. Mates of males lacking the glycoprotein Acp36DE, which is needed for the accumulation of sperm in the storage organs, fail to complete the full sequence of the conformational changes. Our results show that uterine conformational changes are important for proper accumulation of sperm in storage and identify a seminal protein that mediates these changes. Four Acps included in this study, previously shown to affect sperm release from storage (CG9997, CG1656, CG1652, and CG17575), are not necessary for uterine conformational changes to occur. Rather, consistent with their role in later steps of sperm storage, we show here that their presence can affect the outcome of sperm competition situations.

reproduction | seminal protein | sperm competition | sperm storage

Sperm storage is an important process in the sexual reproduction of a multitude of animals—including insects and mammals—with internal fertilization (1). In *Drosophila*, the subset of seminal proteins generated and secreted from the accessory gland of the male reproductive tract, known collectively as ''Acps,'' is essential for sperm storage. Females who receive less than 1% of wild-type levels of Acps during mating store fewer than 10% as many sperm as controls (2), and the few sperm that they do store are not used for fertilization (3). In addition to their role in sperm storage, Acps also mediate physiological and behavioral postmating responses (PMRs) in female *D. melanogaster* (4). Acp-mediated PMRs occur in two distinct phases: a short-term response (STR), which occurs within the first 24 h after the start of mating (ASM), and a long-term response (LTR), which occurs thereafter (5–7). Whereas most Acps are detectable in females only during the short-term period, the Acp called sex-peptide (SP, Acp70A) persists in the female reproductive tract for several days postmating, and has been implicated in the maintenance of the LTR. Its persistence is thought to be due to its binding to the tails of stored sperm. The gradual release of SP from those sperm tails allows it to reach targets and act continually within the female, for as long as sperm are stored (8–12). Thus, sperm storage is instrumental in *D. melanogaster* reproduction and is required for maintenance of the LTR.

Sperm are stored in two types of specialized organ in the *D. melanogaster* female reproductive tract (RT): the single seminal receptacle and a pair of spermathecae. These structures are known collectively as the sperm storage organs (SSOs). Sperm can be stored for up to 2 weeks after mating (13). Storage of sperm has several important consequences. First, it allows females to decouple the processes of mating and fertilization (1). Second, it provides females with the opportunity to store sperm from more than one male. This allows stored sperm from different males to compete for opportunities to fertilize a female's eggs (14) and, potentially, permits sperm preference/choice by the female (15, 16). Third, as noted above, stored sperm are necessary to maintain the long-term PMRs induced by Acps (8–10). The process of sperm storage involves several different steps: the entry of sperm into storage, their retention and maintenance in storage, and their release from storage (1). In *D. melanogaster*, Acps have been shown to be necessary for sperm storage, and individual steps of the process appear to be controlled separately by different Acps (17–21).

In both *Drosophila* and mammals, changes in the shape and/or contraction of the female RT are induced by receipt of seminal fluid. These changes have been hypothesized to assist in sperm movement within the female RT (22–25). In mammals, small molecules in the seminal plasma, such as prostaglandins, have been implicated in affecting the contractility of female RT tissues (26, 27). In *D. melanogaster*, the receipt of Acps (in aggregate) within the female RT has been shown to induce a reproducible sequence of changes in uterine conformation (25). The nature of female RT contraction would seem to have differing functions with regard to sperm movement in these organisms. For example, human sperm are small and must travel a great distance relative to their size. In contrast, *Drosophila* sperm are very long (28)—with *D. melanogaster* sperm measured at 1.7 mm (29), roughly half the length of an adult female—and travel a considerably smaller distance, less than their own length, to reach the storage organs. This would suggest that conformational changes of the *D. melanogaster* uterus may be necessary to aid the movement of these long sperm en masse to the SSOs. The individual Acp(s) responsible for these conformational changes, and the role that these changes play in sperm storage, if any, have yet to be elucidated.

Here, we used RNAi to test whether five Acps known to associate with sperm and/or the SSOs play a role in the postmating uterine conformational changes of the *D. melanogaster* RT. One Acp we tested is Acp36DE, a glycoprotein necessary for normal levels of sperm to accumulate within the SSOs (17, 18). Four other Acps: CG9997, CG1652, CG1656, and CG17575 (a predicted protease, two lectins, and a CRISP, respectively) enter the SSOs (CG9997, CG1652, and CG1656) or associate with the sperm mass (CG17575) and affect the release of sperm from storage (19, 30). That these Acps are detectable in the female RT for only a short period—4 h or less after mating (30)—suggested

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that despite their later effects, their direct action occurs early in reproduction. Consistent with this, recent results indicate that these four Acps are needed for SP to associate with stored sperm (21).

We show here that knockdown of Acp36DE prevents progression past a specific stage of the uterine conformational changes. Failure to progress beyond this stage prevents formation of a discrete sperm mass adjacent to the entrances of the SSOs, a position within the uterus from where sperm enter storage. This provides an explanation for the sperm storage defect that was previously reported for the mates of Acp36DE mutant males (17, 18). Knockdown of the other four Acps did not affect the uterine conformational changes, suggesting that these Acps do not play a role in the earliest stages of sperm storage and that uterine changes are needed only in the initial stages of sperm storage. That these Acps affect later events for stored sperm is extended here by experiments showing that their presence vs. absence affects males' success in defensive sperm competition. This study implicates the *Drosophila* seminal protein Acp36DE in mediating uterine conformational change and suggests that progression through the uterine conformational changes is a requirement for proper sperm movement within the *D. melanogaster* female reproductive tract.

Results and Discussion

Acp36DE Is Necessary for Completion of the Uterine Conformational Changes. Adams and Wolfner (25) classified the mating-induced conformational changes that occur in the female RT into 10 distinct stages. During progression through these stages, the uterus transitions from an S-shaped structure with a closed lumen to a more open and turgid conformation. These changes also expose the entryways of the SSOs to the lumen of the uterus. In the final stages of the conformational changes, the sperm mass is visible adjacent to the openings of the SSOs. The initial stages (1–4) occur rapidly (≈5 min), and the entire process takes about 45 min to complete. To identify Acp(s) that play a role in the conformational changes, we systematically removed individual Acps from the males' ejaculate via RNAi and examined the conformation of the RTs of females mated to knockdown vs. control males of similar genetic backgrounds (see *Materials and Methods*).

For our initial assessment, females mated to knockdown or control males were frozen at −20 °C at 35 min ASM (mating typically lasts ≈20 min). This time point was chosen because in matings to control males it gives a good distribution of the RT conformational stages (see black bars—the controls—in Fig. 1), including some RTs that have progressed to the later conformational stages. Mates of Acp36DE knockdown males failed to reach the final stage of the uterine conformational changes (stage 10); the majority of Acp36DE knockdown mates appeared to have "stalled" in the middle stages (see Fig. 1). This was seen in replicate experiments with two independent lines of males carrying an Acp36DE RNAi transgene, and in additional experiments using Acp36DE knockdown via a different construct, in a different genetic background (see Table S1). These results suggest that Acp36DE is needed for progression of the uterine conformational changes to the final stages by 35 min ASM. In contrast to Acp36DE, mates of males knocked down for CG9997, CG1656, CG1652, or CG17575 showed no difference in conformational stage distribution when compared to mates of control males (see Fig. 1). These latter four Acps were previously shown not to affect accumulation of sperm in storage, but rather, to affect the release of sperm from storage (19). Our observations suggest that the Acp-induced uterine conformational changes do not influence sperm release from storage, suggesting that the role of the conformational changes may be limited to the early phase of sperm storage, in which sperm enter and accumulate in the SSOs.

One possible explanation for the differences in uterine conformation observed between mates of Acp36DE knockdown and control males at 35 min ASM is that mates of Acp36DE knockdown males might need additional time to reach the later stages of uterine conformation. To test this, we examined the RTs of females mated to RNAi vs. control males at later time points. In the absence of Acp36DE, female RTs proceeded to stages 1–4 as normal, arriving at the "hourglass" shape that is typical of stage-5 uteri (see Fig. 2 *A* and *E*) (25). After this stage, RTs of mates of Acp36DE knockdown males did not proceed efficiently, or completely, through the subsequent stages. Even at 60 min ASM (see Fig. 2*D*), a time point by which all mates of control males have completed the conformational changes (reached stage 10) (see Fig. 2*I*) and sperm storage is maximal (18), the RTs of the mates of Acp36DE knockdown males are still arrested.

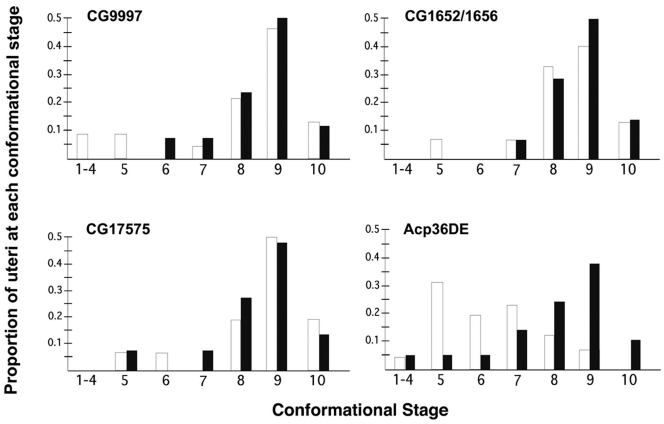

Fig. 1. Distributions of the uterine conformational stages, shown as proportions, at 35 min ASM in mates of knockdown males (white bars) or control males (black bars) for the specified Acp. Mates of males knocked down for CG9997 (Wilcoxon: $Z = 0.3997$, $N_{RNAi} = 24$, $N_{Cont} = 18$, $P < 0.7$), CG1652/1656 (Wilcoxon: $Z = -1.08$, $N_{RNAi} = 16$, $N_{Cont} = 14$, $P < 0.27$), and CG17575 (Wilcoxon: $Z = -0.210$, $N_{RNAi} = 16$, $N_{Cont} = 15$, $P < 0.84$) show no difference in their distributions when compared to mates of control males. Mates of Acp36DE knockdown males (Wilcoxon: $Z = 3.27$, $N_{RNAi} = 26$, $N_{Cont} = 21$, $P < 0.0011$) do not complete the full complement of the conformational changes. The results here, from one replicate of the experiment, are typical of those seen in all replicates (see Table S1).

Thus, our data indicate that Acp36DE is necessary for the uteri of mated females to fully proceed through all of the stages of conformational change and identifies Acp36DE as a seminal protein to be involved in the conformational status of the uterus in *Drosophila*. Initiation of the uterine conformational changes was unaffected by the absence of Acp36DE. Because Acps in aggregate are needed to initiate as well as progress through the conformational changes (25), we expect that other Acp(s) will be found that trigger the changes before the requirement for Acp36DE at stage 5.

Uterine Conformational Changes Are Important for Maximal Sperm Storage. Under normal conditions, at the mid-point of the conformational changes (stage 5), the majority of sperm within the uterus are found below the hourglass constriction. A small subset of sperm proceeds through and beyond the constriction, moving to a position adjacent to the seminal receptacle. A stream of sperm connects the sperm mass, which contains the majority of sperm, to the subset that had proceeded past the constriction. In the later conformational stages, the constriction opens up. The entire uterus takes on an ovoid shape, with sperm ultimately condensing into a single dense, discrete mass that remains just below the openings of the SSOs while the sperm are actively being stored (25). Sperm that are not stored will be

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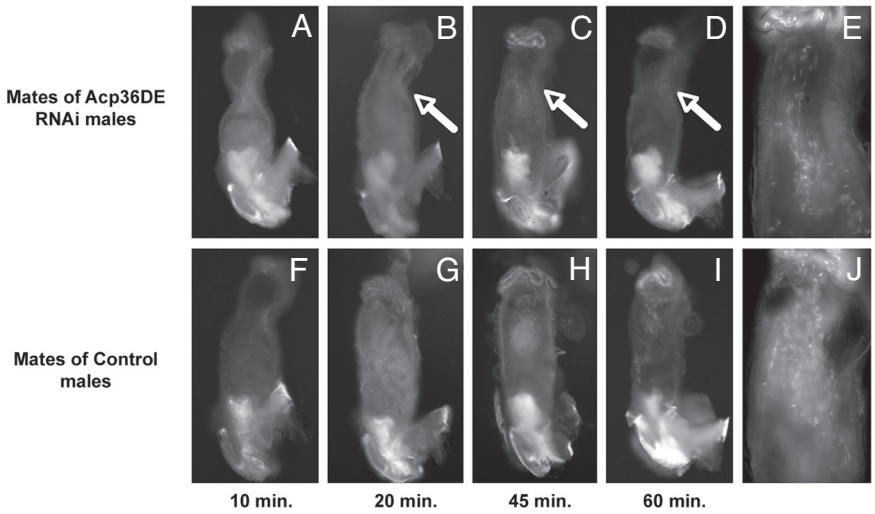

Fig. 2. Uterine conformation of mates of Acp36DE knockdown males (*A–E*) and mates of control males (*F–J*) at the indicated time points. Mates of Acp36DE knockdown males rarely proceed beyond the constriction typically seen in a stage 5 uterus (as in *A*). Sperm that do not proceed beyond the constriction (white arrows) do not form a discrete mass adjacent to the seminal receptacle (as in *I*) and instead remain behind the uterine constriction, becoming more dispersed throughout the lower uterus (*B–D*). A 20× magnification of uteri dissected at 45 min ASM are shown in *E* (mate of Acp36DE knockdown male) and *J* (mate of control male) showing the sperm mass to remain below the uterine constriction in mates of Acp36DE knockdown males. Uteri are positioned anterior (*Top*) to posterior (*Bottom*).

physically pushed out of the uterus during passage of the first egg to be laid after mating (1).

In the absence of Acp36DE, with the conformational stages blocked at the hourglass stage, the sperm did not condense into a single mass. Instead, the sperm mass remained below the hourglass constriction, becoming more dispersed than normal in the posterior uterus (see Fig. 2 *C–E*). Presumably, only the small subset of sperm that move beyond the uterine constriction are actively stored, but the many sperm remaining in the uterus are not, subsequently being expelled during passage of the first egg. The uterus remains in an open conformation until approximately 3–4 h after mating (see Table S2); it then remains closed despite the passage of subsequent eggs. Additionally, virgin females RTs are maintained in a closed conformation until mating (25) even though these females lay eggs. These results would suggest that the opening of the uterus ASM is not needed for the passage of eggs. Instead, its role may be to facilitate processes that accompany the receipt of seminal fluid and/or the storage of sperm.

Previously, we reported that initial sperm entry into the SSOs does not require Acp36DE, but rather that Acp36DE is necessary for accumulation of sperm into the SSOs after initial sperm entry. At 1 h ASM, approximately 50% as many sperm were stored in mates of Acp36DE-null males as compared to mates of controls males (18). The distribution of uterine conformation stages that we see in mates of Acp36DE-knockdown males provides an explanation for this observation: the ability of uteri to open the constriction of the hourglass, and thus to proceed past the mid-point of the shape changes, is necessary to facilitate sperm accumulation into storage. We show this process to be mediated by Acp36DE. Sperm storage in Acp36DE RNAi mates in this study was approximately 60% of that of controls at 1 h ASM (line 1: $N_{Cont.} = 7$, mean sperm stored in SR = 360, $N_{KD} = 10$, mean = 221, $t = -8.63$, $P < 0.001$; line 2: $N_{Cont.} = 7$, mean = 352, $N_{KD} = 7$, mean = 229, $t = -7.66$, $P < 0.001$). We believe that our observation of higher levels of stored sperm in mates of Acp36DE RNAi males relative to mates of null males is most likely due to residual levels of Acp36DE remaining in the knockdown males.

Interestingly, the effects of Acp36DE on the female RT may persist longer than the protein itself. In experiments in which females mate first to an Acp36DE deficient male (or a control male), and subsequently to another (wild-type) male, donation of Acp36DE by the first mating male appeared to facilitate progeny production by the second mating male (31), suggesting that Acp36DE might "prime" the female RT for sperm storage. This priming may reflect Acp36DE's role in mediating uterine conformational changes, as reported here. Perhaps, after having been stimulated by Acp36DE to open, the constricted region of the RT might not completely revert back to virgin state, thus making progression through the conformational stages easier in response to a subsequent mating.

Mating, and receipt of Acps, causes vesicle release from nerve termini in different regions of the RT at different times (32). Additionally, recent reports by Kapelnikov et al. (33, 34) show that mating up-regulates genes and proteins with known roles in muscle and epithelial differentiation in the oviduct (33), and that structural changes occur in the oviduct postmating (34). Analogous changes may occur in the uterus postmating. Our results establish Acp36DE as an intriguing candidate for further study in its potential involvement in these changes.

Acps Affecting Sperm Release Affect the Ability of Sperm To Be Displaced by Rival Male Sperm. Previously, it was shown that Acp36DE affected defensive sperm competition: first-mating $Acp36DE^1$ (null) males sired a significantly smaller proportion of progeny (P1) than did control males (31). Presumably, this occurs indirectly due to Acp36DE's effect on the number of sperm stored, and thus available for sperm competition. Since knockdowns of CG9997, CG1656, CG1652, or CG17575 do not affect initial sperm storage levels at early time points (2 h ASM), but rather lead to the retention of a high number of sperm in storage after several days (19), analogous logic would predict that removal of any of these Acps would affect defensive sperm competition in one of the following ways: 1) sperm from a knockdown male would be present in the female RT in greater numbers than in controls, thus the knockdown male will ultimately sire a higher proportion of progeny than controls following a second mating, or 2) sperm from a knockdown male will be present in larger numbers in the female, but they will not be used by the female (who is unable to release them from storage), so these males will ultimately sire a lower proportion of progeny

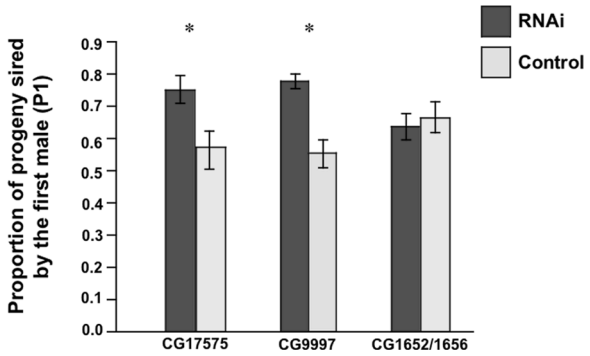

Fig. 3. CG9997 and CG17575 RNAi males sire a higher proportion of progeny than their controls in defensive sperm competition assays carried out on flies that re-mated 3 days after a first-mating. Asterisks mark statistically significant differences. CG9997: Wilcoxon: $Z = 3.7$, $N_{RNAi} = 29$, $N_{Cont} = 33$, $P < 0.002$. CG17575: Wilcoxon: $Z = -2.4$, $N_{RNAi} = 25$, $N_{Cont} = 19$, $P < 0.016$. CG1652/56 knockdown males showed no difference from their controls in defensive sperm competitive ability under these mating-timings (Wilcoxon: $Z = 0.815$, $N_{RNAi} = 25$, $N_{Cont} = 25$, $P < 0.41$), but they did show a significant difference in the same direction when assays were conducted with the second-mating occurring 7 days after the first (see text).

than controls following a second mating. We tested these predictions by assaying CG9997, CG1656/CG1652, and CG17575 knockdown males in defensive sperm competition.

In defensive sperm competition—assessed with a second mating on the third day after the initial mating—mates of CG9997 and CG17575 knockdown males sired a higher proportion of progeny than did control siblings (see P1, Fig. 3). Although we observed no significant difference in P1 of CG1652/56 knockdown vs. control males in experiments of this design, when females were mated to CG1652/56 knockdown vs. control males and then re-mated on the seventh day after the first mating, a small but significant effect was observed in the same direction as the effect seen with CG9997 and CG17575 knockdown males shown in Fig. 3—mates of CG1652/56 knockdown males sired a higher proportion of progeny than controls (mean P1: $N_{RNAi} = 0.06$, $N_{Cont} = 0.01$. Wilcoxon: $Z = -3.84$, $N_{RNAi} = 27$, $N_{Cont} = 25$, $P < 0.0001$). Thus, knockdown of these Acps allows males to sire a higher percentage of progeny than would controls, when they are the first of two males to mate. Given that these Acps appear to act within a network (21), a simple explanation for the more rapid appearance of P1 differences for CG9997 and CG17575 knockdowns vs. for CG1652/56 knockdowns could reflect that CG1652 and CG1656 are gene duplicates and thus might carry out similar functions; it might thus take longer for the mates of CG1652/56 knockdown males to differ enough from mates of controls to show an effect in defensive sperm competition assays. Separately, the results of these experiments also indicate that receipt of wild-type levels of Acps from a second mating male's ejaculate rescues the sperm release defect observed for these Acps (19). This finding agrees with a previous study that showed the sterility of Acp-less males can be rescued by subsequent mating to males who provide wildtype levels of Acps (but not sperm), which Xue and Noll termed "copulation complementation" (3).

Conclusion

Sperm storage contains multiple steps that ensure the efficiency of the process, with these steps regulated by several seminal proteins (Acps) received by the female during mating (17–21). The Acps we studied here are influential in at least two ways: (1) by affecting the number of sperm that get stored (Acp36DE) and (2) by affecting the number of sperm that are retained within the SSOs (19), which could underlie their effects on males' success in sperm competition situations.

We have shown that Acp36DE, which affects the accumulation of sperm in storage, mediates the uterine conformational changes that are initiated in the female RT during mating. Furthermore, we have shown that these changes are necessary to facilitate the storage of sperm. Non-protein components of the male ejaculate had previously been shown known to affect the muscle contraction state of the mammalian female RT (26, 27, 35, 36). Our results identify a seminal fluid protein that regulates postmating uterine conformation, in *Drosophila.* The genetics of this model organism will help to dissect how male and female molecules interact to cause these changes.

Materials and Methods

Fly Strains and Rearing. All flies were maintained on yeast-glucose medium at room temperature (22 ± 1 °C) and a 12:12-h light/dark cycle. To visualize sperm, we crossed an X-linked transgene encoding protamine-GFP (a generous gift of John Belote and Scott Pitnick, Syracuse University) into the *tubulin*-GAL4/*TM3*, *Sb* line (stock no. BL5138; Bloomington Stock Center), generating a protamine-GFP; *tubulin*-GAL4/*TM3*, *Sb*, *Ser* stock. This stock was crossed to flies homozygous for a UAS-driven knockdown construct to generate knockdown and control flies as described below. Assays were done by crossing knockdown and control males to females of the Canton-S strain of *D. melanogaster*.

Generation of RNAi Lines. To assess the effects of knockdown of Acp36DE, CG9997, CG1656, CG1652, and CG17575 on uterine conformation, we generated RNAi and control males by crossing female *tubulin*-GAL4/*TM3*, *Sb* (Bloomington) to the UAS-RNAi generating homozygous males. For CG9997, CG1656, CG1652, and CG17575, SympUAST-GW-Acp males reported by Ravi Ram et al. (37) and Ravi Ram and Wolfner (19) were used. The Acps CG1656 and CG1652 are gene duplicates; knockdown for the targeted Acp also knocks down its duplicate (19). To knock down Acp36DE, we generated a SympUAST-GW fly line as in Ravi Ram et al. (37), targeting a 96-bp sequence of Acp36DE's 3′UTR (beginning and ending: GTTAACCTTA…ATAAAATAAT). Cloning, vectors, and transgenes were as in Ravi Ram and Wolfner (19). An additional RNAi-generating line for Acp36DE was obtained from the Vienna Drosophila RNAi Center (construct ID: 37993) (38). Knockdowns were confirmed by Western blotting as in Ravi Ram et al. (34).

To visualize sperm in Acp36DE RNAi males, we generated knockdown and control males with GFP-labeled sperm by crossing protamine-GFP; *tubulin*-GAL4/*TM3*, *Sb*, *Ser* females to SympUAST-GW-Acp36DE homozygous males. Males and females were collected within 4 h of eclosion to ensure that they were unmated, and aged separately for 3–5 days in vials containing yeast-glucose medium before experimentation.

Uterine Conformational Assays and Dissections. Using an aspirator, an individual male and female were placed together into a glass vial containing moistened Whatman 3MM filter paper. The time at which individual pairs started to mate was recorded. Mating pairs were frozen at −20 °C at the indicated time after the start of mating (ASM). Flies were maintained frozen for 24–48 h before dissection. Dissections were conducted as previously described (25) in physiological saline (0.7% NaCl). Uteri were mounted in halocarbon oil and visualized without a coverslip using a Leica DM 500B fluorescence microscope (Leica Microsystems). For the uterine conformational assays, the total number of flies examined, and the number at each conformational stage are given in Table S1. JMP software was used for statistical analysis (JMP IN, 5.1.2), with stage distribution analysis of knockdown vs. control mates using a Wilcoxon test (rank sums).

Sperm Competition Assays. Defensive sperm competition assays (experimental male is the first to mate) were done as in Clark et al. (39) using a *cn bw* strain of *D. melanogaster* (*cn bw I*). Briefly, *cn bw* virgin females were aged 3–5 days and crossed to knockdown males (*cn bw* females were crossed to sibling, control males in parallel). Females were individually placed into a vial containing yeast-glucose medium and given the opportunity to re-mate on the third day to a single *cn bw* male; matings were observed. Upon re-mating, the female was placed into a fresh food vial and allowed to lay eggs for 10 days. Paternity of the progeny was determined using eye color. Replicates were done using a *cn bw* line that had been back crossed to the Oregon-R strain of *D. melanogaster* for 10 generations (*cn bw II*; kindly provided to us by Anthony Fiumera, Binghamton University). JMP software was used for statistical anal-

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ysis (JMP IN, 5.1.2). P1 measurements (the proportion of progeny sired by the first mating male) were analyzed using a Wilcoxon test (rank sums).

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