Retention of Ejaculate by *Drosophila melanogaster* Females Requires the Male-Derived Mating Plug Protein PEBme

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ABSTRACT Within the mated reproductive tracts of females of many taxa, seminal fluid proteins (SFPs) coagulate into a structure known as the *mating plug* (MP). MPs have diverse roles, including preventing female remating, altering female receptivity postmating, and being necessary for mated females to successfully store sperm. The *Drosophila melanogaster* MP, which is maintained in the mated female for several hours postmating, is comprised of a posterior MP (PMP) that forms quickly after mating begins and an anterior MP (AMP) that forms later. The PMP is composed of seminal proteins from the ejaculatory bulb (EB) of the male reproductive tract. To examine the role of the PMP protein PEBme in *D. melanogaster* reproduction, we identified an EB GAL4 driver and used it to target *PEBme* for RNA interference (RNAi) knockdown. *PEBme* knockdown in males compromised PMP coagulation in their mates and resulted in a significant reduction in female fertility, adversely affecting postmating uterine conformation, sperm storage, mating refractoriness, egg laying, and progeny generation. These defects resulted from the inability of females to retain the ejaculate in their reproductive tracts after mating. The uncoagulated MP impaired uncoupling by the knockdown male, and when he ultimately uncoupled, the ejaculate was often pulled out of the female. Thus, PEBme and MP coagulation are required for optimal fertility in *D. melanogaster*. Given the importance of the PMP for fertility, we identified additional MP proteins by mass spectrometry and found fertility functions for two of them. Our results highlight the importance of the MP and the proteins that comprise it in reproduction and suggest that in *Drosophila* the PMP is required to retain the ejaculate within the female reproductive tract, ensuring the storage of sperm by mated females.

KEYWORDS mating plug; sperm storage; PEBme; *Drosophila* reproduction

IN numerous species comprising diverse taxa, a solidified structure forms inside the female reproductive tract during (or shortly after) mating that is referred to as the *mating plug* (MP; also called the *copulatory plug*; we will refer to these structures collectively as MPs). MPs are largely a coagulation of male seminal fluid components. In species that produce a MP, its role in reproduction varies. In some species, MP formation is thought to guard against sperm competition. For example, in primates, MPs are seen most often in species whose females mate multiply (Dixson and Anderson 2002). Primate MPs have been suggested to prevent remating (Dorus *et al.* 2004), thus acting as a form of passive mate guarding (Dunham and Rudolf 2009). In the mouse, perturbing (Murer *et al.* 2001) or preventing (Dean 2013) MP formation reduces male fertility; in the absence of MP formation, sperm migration to the sites of fertilization is impaired (Dean 2013), suggesting that the mouse MP is important for proper sperm function.

MPs are common in insects [reviewed in Avila *et al.* (2011)]. Insect MPs have a variety of functions that affect fertility, from altering female receptivity postmating to being required for sperm storage in mated females. For example, *Drosophila hibisci* and *D. melanogaster* MPs reduce female

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receptivity in the short term (Polak *et al.* 1998; Bretman *et al.* 2010). In bumblebees, MPs physically switch off receptivity (Baer *et al.* 2001; Sauter *et al.* 2001) and have functions related to sperm competition (Duvoisin *et al.* 1999). In the malaria mosquito *Anopheles gambiae*, the MP is necessary for sperm storage (Rogers *et al.* 2009) and has been proposed to contribute to immunity and *Plasmodium* transmission (Mitchell *et al.* 2015).

In species whose MP biochemistry has been examined, MPs are generally composed of male-derived seminal fluid proteins (SFPs) (Lung and Wolfner 2001; Kawano and Yoshida 2007; Rogers *et al.* 2009; Bretman *et al.* 2010; Dottorini *et al.* 2012; Dean 2013). Upon transfer, SFPs effect numerous physiological and behavioral changes in mated females [reviewed in Poiani (2006) and Avila *et al.* (2011)]. However, female-derived proteins also have been found in MPs (Rogers *et al.* 2009). We know little about the role of MPs, and most of the SFPs that comprise them, in reproduction. Here we used the model organism *D. melanogaster* to dissect the role of MPs in postmating events in this species.

D. melanogaster MPs form shortly after mating begins (Neubaum and Wolfner 1999; Lung and Wolfner 2001). They have two distinct regions comprised of SFPs originating from different male reproductive tissues. A dense posterior mating plug (PMP) composed of SFPs from the ejaculatory bulb (EB) (Lung and Wolfner 2001; Bretman *et al.* 2010) forms ~5 min after the start of mating (ASM). A gelatinous anterior mating plug (AMP) primarily composed of SFPs from the male accessory gland (Bertram *et al.* 1996; Lung and Wolfner 2001) forms ~20 min ASM. The MP remains in the uterus for several hours until females eject the structure along with unstored sperm (*e.g.*, excess sperm from the recent mate and displaced sperm from previous mates) (Manier *et al.* 2010; Lüpold *et al.* 2013; Lee *et al.* 2015). A recent study identified female innervation that modulates MP ejection and showed that this behavior has an impact on fertility (Lee *et al.* 2015). MP ejection may be important in postcopulatory sexual selection because the timing of ejection influences the fertilization success of competing males (Lüpold *et al.* 2013).

Some functions of the *D. melanogaster* AMP are known. Females that do not receive the SFP Acp36DE during mating do not form an AMP (Bertram *et al.* 1996) and fail to store sperm at optimal levels (Neubaum and Wolfner 1999; Bloch Qazi and Wolfner 2003). Further, Acp36DE induces conformational changes in the female's uterus that are important for efficient sperm storage (Avila and Wolfner 2009). Whether the presence of Acp36DE in the AMP or its localization to sperm and/or other female tissues (Bertram *et al.* 1996) is required for its sperm storage function is unknown.

Less is known about the PMP. Two PMP proteins have been identified previously: protein of the ejaculatory bulb of *melanogaster* (PEBme) (Ludwig *et al.* 1991; Lung and Wolfner 2001) and protein of the ejaculatory bulb II (PEBII) (Bretman *et al.* 2010). While the function of PEBme is unknown, PEBII reduces the likelihood of female remating in the first 4 hr postmating (Bretman *et al.* 2010). However, additional functions of the PMP in general, and of PEBme specifically, are not well understood.

We report that PEBme is essential for complete male fertility: it is required for PMP integrity and retention of the ejaculate in mated females. RNA interference (RNAi) knockdown of *PEBme* causes defects in early sperm storage events, suppressing the number of sperm in storage by 24 hr ASM, and also seriously impairs other postmating responses. Further, when *PEBme* knockdown males disengage from females at the end of mating, the ejaculate adheres to their genitalia, resulting in ejaculate loss from the female reproductive tract. Our results show that PEBme is necessary for proper PMP coagulation and to retain ejaculate within the uteri of mated females to ensure maximal sperm storage. Finally, we identified 60 additional MP proteins by mass spectrometry and showed that two of the most abundant are also required for full fertility. The results reported here show that the *D. melanogaster* MP is integral for optimal fertility.

Materials and Methods

Fly stocks

We used the UAS;GAL4 system (Brand and Perrimon 1993) to express transgenic constructs (under UAS control) in the specified tissue. *CrebA*-GAL4 [Bloomington *Drosophila* Stock Center (BDSC) #49409] was used to express mCD8::GFP (BDSC #5137), $Rh1^{G69D}$ (Ryoo *et al.* 2007), and *PEBme* double-stranded RNA (dsRNA) [two independent Vienna *Drosophila* RNAi Center (VDRC) lines: #100183 and #18973] in male EBs. Also, *ovulin*-GAL4 (Chapman *et al.* 2003) was used to express CG8626 and CG15616 dsRNA (VDRC #103960 and #105778, respectively) in male accessory glands. All males were mated to Canton-S females. In our $Rh1^{G69D}$ experiments, *CyO* balancer siblings were used as controls. In our *PEBme*, *CG15616*, and *CG8626* experiments, control males were generated by crossing the *CrebA*-GAL4 driver to *attP2* females (VDRC #60100). Knockdowns were quantified by RT-PCR (Supporting Information, Figure S1); the data illustrated that *CrebA*-GAL4 is an effective driver for RNAi in the EB. Flies were raised at 23° on standard yeast-glucose medium and a 12:12-hr light:dark cycle and aged 3–5 days before use in each experiment.

Microscopy

Male reproductive tract expression of *CrebA*-GAL4 was determined by driving membrane-bound mCD8::GFP (Lee and Luo 1999). Reproductive tracts were dissected from UAS-*mCD8*::GFP/+; *CrebA*-GAL4/+ males and visualized for GFP expression. To examine MP formation, lower reproductive tracts (*i.e.*, uterus, sperm storage organs, and common oviduct) of females mated to experimental or control males were dissected and visualized under ultraviolet (UV) illumination to take advantage of PEBme's autofluorescence (Lung and Wolfner 2001). Tissues were dissected in 1× PBS and visualized using a Leica DM 500B fluorescence microscope (Leica Microsystems).

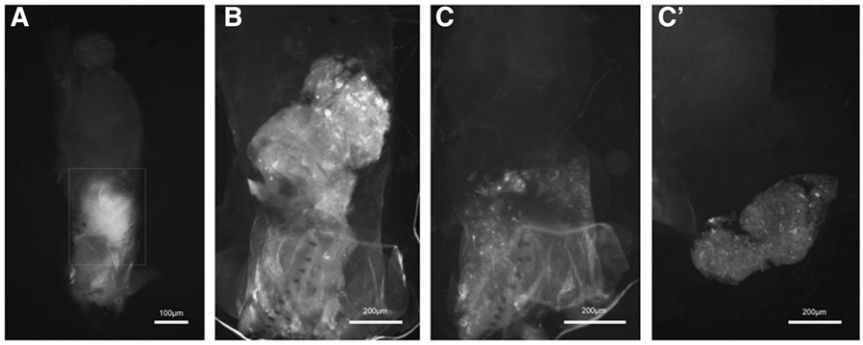

Figure 1 *PEBme* is required for proper PMP formation. PMPs in mates of control males are fully formed (A and B) at 35 min ASM. PMPs in mates of *CrebA* > *PEBme*^{RNAi} males are not coagulated (C) or are reduced (C′) at this time. In an additional experiment to obtain quantitation, we observed that among 15 females mated to *CrebA* > *PEBme*^{RNAi} males, ~33% had uncoagulated MPs, ~33% had reduced MPs, and ~33% had MPs that looked normal (to this resolution). To see the PMP in the context of the uterus, the reproductive tract in A was not placed under a coverslip (the dotted box highlights the PMP). To better visualize the PMP, uteri in B and C′ were placed under a coverslip. B and C′ are at the same magnification. PMPs are visualized under UV illumination.

Fertility assays and interrupted matings

Fertility (progeny) and fecundity (eggs laid) assays were performed as in Herndon and Wolfner (1995). Females used in our fertility assays were all mated on the same day, and at the same time. We counted the number of eggs and resulting progeny for each female for each day of the experiment. Comparisons of egg and progeny production of the 5-day assays were performed using a repeated-measures ANOVA in JMP 9.02. This allows for the study of a single individual over a longitudinal period (*i.e.*, a single female and how her egg laying/progeny production changes over time). To interrupt matings, a vial containing the mating pair was lightly flicked with a finger at 13 min ASM; such agitation caused control as well as experimental pairs to uncouple. Males then were immediately removed.

Uterine conformation assays

Uterine conformation assays were performed as in Avila and Wolfner (2009). Females mated to experimental or control males were frozen in liquid nitrogen at 35 min ASM. To obtain sufficiently large sample sizes, females from two to three independent mating events were used. Uteri were dissected in 1× PBS, visualized with an Olympus SZ61 dissection microscope, and staged as in Adams and Wolfner (2007). For each female, the stage of uterine conformation was determined, and the distribution of stages in females mated to experimental males, compared to mates of control males, was analyzed using a Wilcoxon test (rank sum) in JMP 9.02.

Sperm counts

Females were mated to experimental or control males (matings were observed), frozen in liquid nitrogen at 35 min or 24 hr ASM, and stored at −80° until reproductive tracts were prepared for sperm counts. To obtain sufficiently large sample sizes, females from two to three independent mating events were used. Sperm cells were stained and counted as in Avila *et al.* (2010) using a Zeiss 47 30 11-9901 stereomicroscope. Samples were blind coded before counting. Counts were analyzed using Wilcoxon tests (rank sums) in JMP 9.02.

Receptivity assays

Receptivity assays were done as in Ravi Ram and Wolfner (2007). Females singly mated to experimental or control males were assessed at 24 hr and 4 days ASM for their willingness to remate with a Canton-S male in a 1 hr window. Remating rate was analyzed using Wilcoxon tests (rank sums) in JMP 9.02.

Mass spectrometry

Mated females were frozen in liquid nitrogen at 1 hr ASM to ensure complete MP formation. One-hundred whole PMPs and the posterior half of the AMP (to minimize sperm contamination) were dissected in 1× PBS and then placed in 100 μl 6 M guanidine, 50 mM Tris, pH 7.8, and 0.5% Triton X-100. MPs were solubilized by three rounds of sonication for 30 sec using a Bioruptor (Diagenode) and boiling for 5 min. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was performed at the Cornell Biotechnology Resource Center Proteomics and Mass Spectrometry Facility. Mass spectra were searched against the annotated *D. melanogaster* genome. Identified proteins were ranked by calculating the mean normalized spectral abundance factor as in Kelleher *et al.* (2009).

RNA extraction, video recording, and mating duration methods can be found in the Supporting Information, File S5.

Results and Discussion

CrebA-GAL4 allows knockdown of PEBme

To determine the role of the PMP in *D. melanogaster* reproduction, we targeted the major EB protein PEBme (Ludwig *et al.* 1991; Lung and Wolfner 2001). Numerous male reproductive tract GAL4 drivers were available, but none drove specific expression in the EB. Ubiquitous RNAi-mediated knockdown of *PEBme* using a *tubulin*-GAL4 driver resulted in lethality. Therefore, we screened through Janelia Farm Fly Light GAL4 lines (Pfeiffer *et al.* 2008; Jenett *et al.* 2012) for EB expression. We identified one line that drove expression in the EB and ejaculatory duct of the male reproductive tract (Figure S2). This *CrebA*-GAL4 driver

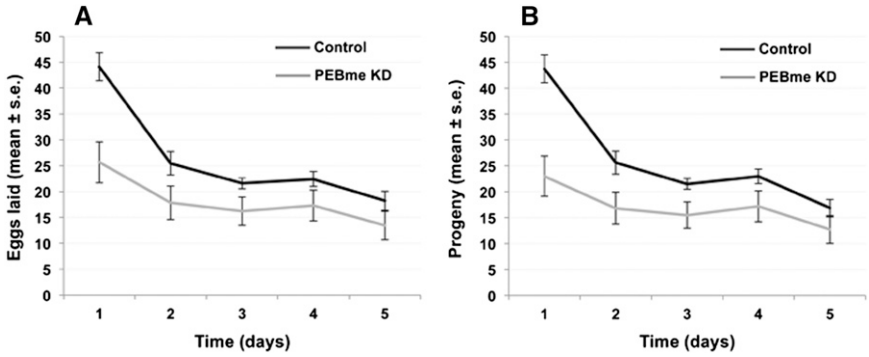

Figure 2 *PEBme* knockdown decreases fertility. Mates of *PEBme* knockdown males (A) lay significantly fewer eggs (N_{PEB} = 18, N_{Cont} = 19, d.f. = 1, F = 7.42, P = 0.01) and (B) produce significantly fewer progeny than mates of control males in the first 5 days after mating (N_{PEB} = 18, N_{Cont} = 19, d.f. = 1, F = 9.12, P = 0.0038).

contains sequence fragments from flanking noncoding regions of *cyclic-AMP response element binding protein A* (*CrebA*), a gene that regulates secretory capacity in *Drosophila* (Fox *et al.* 2010).

We used *CrebA*-GAL4 to induce *PEBme* knockdown in males and examined PMP formation in their mates. Results described here and in the following section are for *PEBme* knockdown with VDRC line #100183; we obtained similar results with an independent VDRC line (#18973) (Figure S3). At 35 min ASM, when the PMP is typically fully formed, many mates of *CrebA* > *PEBme*^{RNAi} males had uncoagulated PMPs (Figure 1C) or PMPs that were reduced in size (Figure 1C′). In cases where the PMP did not coagulate, we observed fluorescent particles in the uterus, suggesting that *CrebA* > *PEBme*^{RNAi} males transferred some PEBme to females, possibly due to incomplete knockdown. However, these particles spread out under the weight of a coverslip (Figure 1C), indicating that the PMPs had not solidified. These findings suggest that PEBme is an essential component for PMP coagulation within the female reproductive tract.

The role of PEBme in MP coagulation is intriguing given PEBme's sequence (Lung and Wolfner 2001). PEBme contains repetitive PGG motifs, similar to those that facilitate self-interaction in proteins that form homopolymers, such as preCol-D of mussel byssal threads (Qin *et al.* 1997) and the flagelliform gland silk protein of spiders (Hayashi and Lewis 1998; Hayashi and Lewis 2001). This suggests a possible mechanism for the role of PEBme in MP coagulation that will be a fruitful avenue for future investigation.

PEBme knockdown reduces the fertility of mating pairs

We assessed egg laying and progeny production when PMP formation was impaired by *PEBme* knockdown. Mates of *CrebA* > *PEBme*^{RNAi} males laid significantly fewer eggs (Figure 2A) and generated significantly fewer progeny (Figure 2B) than mates of control males. Thus, impairing PMP formation by knocking down *PEBme* adversely affects reproductive success.

PEBme knockdown affects sperm storage and female willingness to remate

MP formation influences sperm storage in *D. hibisci* and *A. gambiae* (Polak *et al.* 1998; Rogers *et al.* 2009). Because PMP formation requires PEBme, we asked whether PEBme is also required for sperm storage in *D. melanogaster* females. Uteri of *D. melanogaster* females undergo a series of conformational changes upon SFP receipt—from a closed conformation to completely open (Adams and Wolfner 2007). These changes are necessary for sperm to be stored at maximal levels (Avila and Wolfner 2009). We found that disrupting PMP formation affected postmating uterine conformation. By 35 min ASM, uteri are typically in the final conformational stages (Adams and Wolfner 2007; Avila and Wolfner 2009). In mates of *CrebA* > *PEBme*^{RNAi} males, however, a large proportion of uteri remained closed or failed to open completely (Figure 3A).

Because a significant proportion of mates of *PEBme* knockdown males were defective in progression through the uterine shape changes, they were not expected to accumulate sperm into storage at wild-type levels. We examined sperm numbers in both types of storage organ (the seminal receptacle and the spermathecae) in these females. At 35 min ASM, shortly after females begin active sperm storage (Manier *et al.* 2010), mates of *CrebA* > *PEBme*^{RNAi} males did not differ significantly from controls in sperm accumulation into the seminal receptacle (Figure 3B) or spermathecae (Figure 3B), having similar levels of sperm accumulation overall (Figure 3B). However, we observed high variation in the number of sperm cells stored at 35 min ASM in mates of *CrebA* > *PEBme*^{RNAi} males. Since this time point may not have been sufficient to observe the totality of sperm storage defects, we also examined sperm storage at 24 hr ASM, when defects from *PEBme* knockdown should be readily detected. At 24 hr ASM, mates of *CrebA* > *PEBme*^{RNAi} males contained significantly fewer stored sperm cells than mates of controls in the seminal receptacle (Figure 3C) and spermathecae (Figure 3C); mates of *CrebA* > *PEBme*^{RNAi} males had stored only ~43% as much sperm compared as did controls (Figure 3C). These results suggest that PEBme and, by extension, the PMP is required for sperm to be stored at wild-type levels.

Female refractoriness is affected by knockdown of the PMP protein PEBII (Bretman *et al.* 2010) and can influence male success in competitive situations. Female receptivity decreases after a single mating due to the action of the SFP sex peptide. Sex peptide is retained in females by binding to

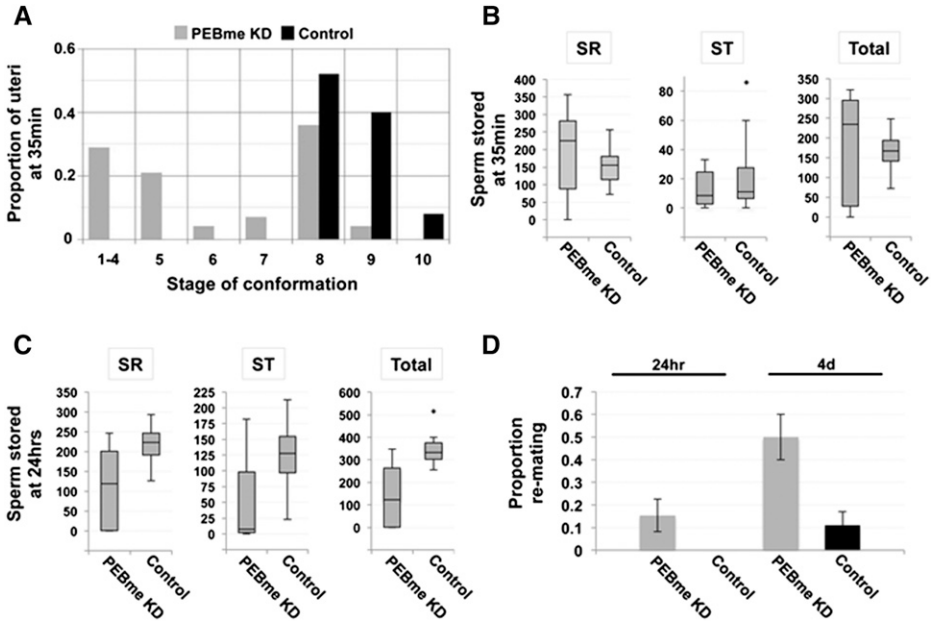

Figure 3 *PEBme* knockdown affects sperm storage processes and female remating. (A) Progression of the uterine conformational changes in mates of *PEBme* knockdown males differs significantly from that of controls (N_{PEB} = 28, N_{Cont} = 25, Z = 4.99, $P <$ 0.0001). Stages 1–4 (closed) are placed into a single group for simplicity. (B) In mates of *PEBme* knockdown males, sperm accumulation into storage did not differ from that of controls at 35 min ASM (SR: N_{PEB} = 21, N_{Cont} = 16, $Z = -1.61$, P = 0.11; ST: N_{PEB} = 14, N_{Cont} = 15, $Z = -0.79$, P = 0.43; total: N_{PEB} = 14, N_{Cont} = 15, Z = 0.72, P = 0.46). (C) By 24 hr ASM, sperm in storage was reduced in mates of *PEBme* knockdown males compared to controls (SR: N_{PEB} = 22, N_{Cont} = 17, Z = 3.26, P = 0.001; ST: N_{PEB} = 16, N_{Cont} = 16, Z = 3.11, P = 0.0017; total: N_{PEB} = 16, N_{Cont} = 16, Z = 3.77, P = 0.0002**)**. (D) Female remating rate is significantly higher by 4 days ASM after mating with a *PEBme* knockdown male rather than with a control male (24 hr: N_{PEB} = 26, N_{Cont} = 22, $Z = -1.88$, P = 0.06; 4 days: N_{PEB} = 26, N_{Cont} = 27, Z = 3.04, P = 0.0023). For the box plots in B and C, the middle horizontal line represents the median, the lower and upper margins of the box represent the 25th and 75th quartiles, and the whiskers extend to the minimum and maximum of the data (excluding potential outliers, which are shown as points outside the whiskers). SR, seminal receptacle; ST, spermathecae; total, SR + ST.

sperm and is slowly released into the female's circulation, altering her behavior (Liu and Kubli 2003; Peng *et al.* 2005; Ravi Ram and Wolfner 2009). Thus, decreased receptivity persists when females contain stored sperm. Since female refractoriness can affect progeny outcomes, particularly in situations of sperm competition, we tested whether knockdown of *PEBme* affected female refractoriness. At 24 hr ASM, we observed a slight, but not significant, rise in female remating after initially mating to *CrebA* > *PEBme*^{RNAi} males compared to controls (Figure 3D). However, by 4 days ASM, females were significantly more likely to remate (relative to controls) if they did not receive PEBme (Figure 3D). The decrease in sexual refractoriness after mating with *PEBme* knockdown males likely results from the reduced amount of stored sperm in these females. Thus, PEBme is required for normal sperm storage and to reduce female receptivity after mating.

PEBme is required to maintain the ejaculate within the female reproductive tract

The severe and varied fertility effects caused by knockdown of the single MP protein PEBme contrast with the more limited phenotype reported for knockdown of *PEBII* (the only other MP protein tested in this way to date). *PEBII* knockdown affects remating but causes no observable defects in egg laying and progeny production by the mates of knockdown males (Bretman *et al.* 2010). This comparison suggested that the breadth of effects on *PEBme* knockdown might reflect loss of functions of many proteins, perhaps by the presence of less ejaculate remaining in the female.

We therefore examined the fate of the ejaculate in mates of *PEBme* knockdown males. We observed that *CrebA* > *PEBme*^{RNAi} males often had difficulty uncoupling from females at the end of mating and that these males remained physically attached to females via the ejaculate for ~10–20 sec (Figure 4A and File S1 and File S2). Often, when *CrebA* > *PEBme*^{RNAi} males detached from females, the ejaculate protruded from the female's posterior (Figure 4B and File S2). When documenting this phenotype, we observed a male incidentally pulling the ejaculate from the female without remaining attached to her (File S3), suggesting that the phenotype may have occurred even when males did not appear to be "stuck" to females following mating. We never observed these phenotypes with control males (File S4). Thus, the decreased fertility of *CrebA* > *PEBme*^{RNAi} males is likely due to the inability of their mates to retain the ejaculate within their uteri when the males uncouple. These observations, in conjunction with the data in Figure 1, suggest that PEBme is needed for the ejaculate to coagulate and thereby to allow a "clean" uncoupling at the end of mating and for female ejaculate retention.

Physical agitation exacerbates the fertility defects of CrebA > PEBme^{RNAi} *males*

Given that ejaculate was incidentally pulled from females when they and their *CrebA* > *PEBme*^{RNAi} mates uncoupled, we wondered whether physically agitating mating pairs would increase the occurrence of this effect and further reduce fertility. To test this, we agitated mating pairs by interrupting mating at 13 min ASM, after sperm and SFPs are transferred to females; SFP and sperm transfers occur at 3 and 8–10 min ASM, respectively (Gilchrist and Partridge 2000; Lung and Wolfner 2001; Manier *et al.* 2010). After interrupted matings, mates of *CrebA* > *PEBme*^{RNAi} males laid

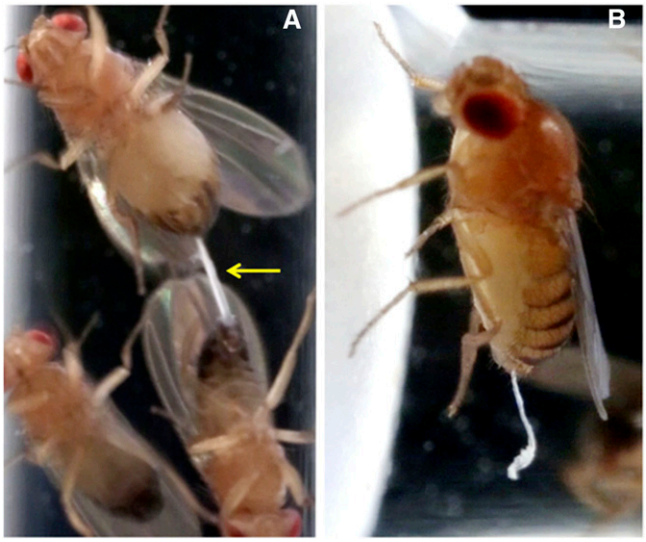

Figure 4 PEBme is required to retain the ejaculate in the female reproductive tract. Video screen captures of *PEBme* knockdown and control males disengaging from females after mating ends: (A) PEBme reduction results in mating males and females becoming "stuck" together via the ejaculate (yellow arrow), (B) often resulting in the ejaculate protruding from the female when males eventually uncouple. N_{PEB} = 8, N_{Cont} = 4.

significantly fewer eggs (Figure 5A) and generated significantly fewer progeny (Figure 5B) than when mating was uninterrupted (Figure 2, A and B).

Interrupting mating did not affect the progression of the uterine conformational changes in mates of control males (Figure S4), indicating that these changes occur normally on SFP transfer during mating. As in uninterrupted matings, progression of the uterine changes significantly differed between mates of *CrebA* > *PEBme*^{RNAi} males and mates of controls when mating was interrupted (Figure 5C). However, interrupting mating significantly exacerbated this effect—progression of the uterine changes in mates of *CrebA* > *PEBme*^{RNAi} males was significantly worse than when mating was uninterrupted (Figure 5D). Finally, interrupting mating affected sperm accumulation in the seminal receptacle and spermathecae at 35 min ASM (Figure 5E), leading to only ~50% of sperm in storage in mates of *CrebA* > *PEBme*^{RNAi} males compared to controls (Figure 5E). Our results show that interrupting mating after sperm and SFP transfer exacerbates the fertility defects associated with *PEBme* knockdown.

Blocking translation of EB proteins affects PMP formation and fertility

Our results show that removal of one EB protein, PEBme, decreases fertility. However, the EB secretes additional PMP proteins (Bretman *et al.* 2010). To determine whether blocking translation of all EB proteins gave more severe fertility defects compared to those observed after knocking down *PEBme*, we drove expression of misfolded rhodopsin (*Rh1*^{G69D}) in the EB. Expressing *Rh1*^{G69D} in secretory tissues causes high levels of endoplasmic reticulum (ER) stress, which blocks translation by inducing the unfolded protein response (Ryoo *et al.* 2007). Driving expression of Rh1^{G69D} in the male accessory gland—the major site of *Drosophila* SFP synthesis—blocks translation and secretion of SFPs from this tissue (Chow *et al.* 2015). Expressing Rh1^{G69D} in the EB similarly blocks the function of this tissue.

In mates of *CrebA* > *Rh1*^{G69D} males, the PMP did not coagulate or was reduced in size (Figure S5). Mates of *CrebA* > *Rh1*^{G69D} males laid significantly fewer eggs (Figure S6A) and produced significantly fewer progeny (Figure S6B) compared to mates of control males. Additionally, in mates of *CrebA* > *Rh1*^{G69D} males, progression of the uterine stages lagged significantly behind that of controls (Figure S6C), and significantly less sperm was stored in the seminal receptacle (Figure S6D) and spermathecae (Figure S5D) at 24 hr ASM, leading to an overall reduction in total sperm stored (Figure S6D).

Surprisingly, the magnitude of the fertility defects in mates of *CrebA* > *Rh1*^{G69D} males was not as severe as that in mates of *CrebA* > *PEBme*^{RNAi} males. We noted that, in contrast to *CrebA* > *PEBme*^{RNAi} males, the mating duration of *CrebA* > *Rh1*^{G69D} males was significantly longer than that of control males (Figure S7, A and B), with 15% of males mating an abnormally long time (>38 min) (Figure S7A). *CrebA*-GAL4 drives expression in some non-EB tissues (Jenett *et al.* 2012), making it possible that inducing ER stress in nonreproductive tissues affected the mating duration of *CrebA* > *Rh1*^{G69D} males. We hypothesize that the additional time that these males mated attenuated the fertility defects associated with impairing PMP formation. These experiments confirm that EB proteins are required for proper PMP formation. The reduced fertility observed in mates of *CrebA* > *PEBme*^{RNAi} and *CrebA* > *Rh1*^{G69D} males likely stems from perturbing PMP formation in their mates, suggesting that proper PMP formation is required for optimal *Drosophila* fertility.

Identification of additional MP proteins

To date, only three *D. melanogaster* MP proteins have been identified: the PMP proteins PEBme and PEBII (Ludwig *et al.* 1991; Lung and Wolfner 2001; Bretman *et al.* 2010) and the AMP protein Acp36DE (Bertram *et al.* 1996). Of these, Acp36DE (Neubaum and Wolfner 1999; Bloch Qazi and Wolfner 2003; Avila and Wolfner 2009) and PEBme (this report) have an impact on female fertility. Because >200 SFPs are transferred to females during mating (Findlay *et al.* 2008, 2009; Yamamoto and Takemori 2010), we wished to identify additional MP proteins that may be important in *Drosophila* reproduction. We analyzed the MP by LC-MS/MS and identified 60 annotated *D. melanogaster* MP proteins (after eliminating four sperm-specific proteins) (Wasbrough *et al.* 2010); the 25 most abundant MP proteins are shown in Table S1. A sizable portion of the MP proteins identified are products of the male accessory glands (flyatlas.org). That PMP formation occurs after genetic ablation of the accessory glands (Xue and Noll 2000) or their products

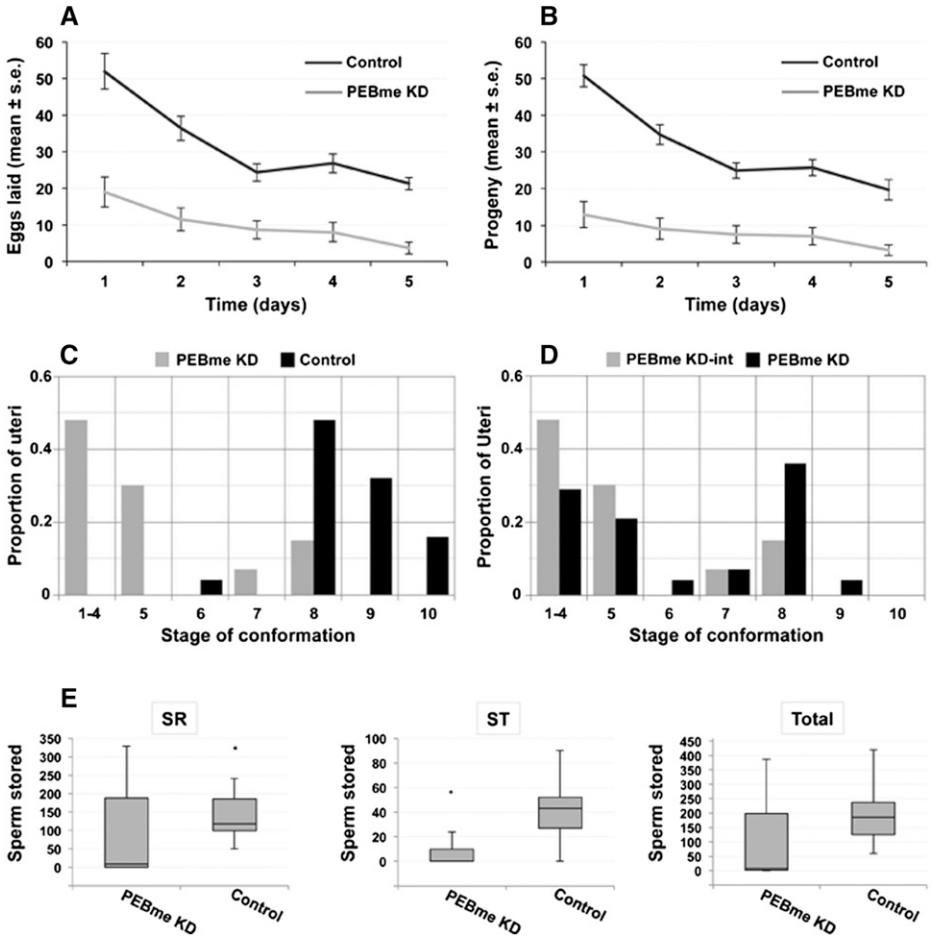

Figure 5 Interrupting mating exacerbates the fertility defects of *PEBme* knockdown. Interrupting mating after sperm and SFP transfer further reduces (A) the number of eggs laid (N_{PEB} = 13, N_{Cont} = 15, d.f. = 1, F = 51.86, $P < 0.0001$) and (B) the progeny produced (N_{PEB} = 13, N_{Cont} = 15, d.f. = 1, F = 66.55, $P < 0.0001$) of females mated to *PEBme* knockdown males compared to controls. Interrupting mating (C) perturbs progression of the uterine conformational changes ($N_{PEB\text{-}int}$ = 27, $N_{Cont\text{-}int}$ = 25, Z = 5.77, $P < 0.0001$) and (D) exacerbates the effect seen in mates of *PEBme* knockdown males when mating was uninterrupted ($N_{PEB\text{-}int}$ = 27, N_{PEB} = 28, Z = −2.07, P = 0.038), (E) leading to a significant reduction in sperm accumulation into storage at 35 min ASM compared to controls (represented as box plots; SR: $N_{PEB\text{-}int}$ = 17, $N_{Cont\text{-}int}$ = 17, Z = 2.10, P = 0.035; ST: $N_{PEB\text{-}int}$ = 17, $N_{Cont\text{-}int}$ = 17, Z = 3.54, P = 0.004; total: $N_{PEB\text{-}int}$ = 17, $N_{Cont\text{-}int}$ = 17, Z = 2.38, P = 0.017). SR, seminal receptacle; ST, spermathecae; total, SR + ST.

(Chow *et al.* 2015) suggests that the accessory gland proteins identified are part of the AMP. SFPs with functions unrelated to the MP have been observed in the AMP: in experiments using sex-peptide::GFP fusions, GFP signal is detected throughout the AMP (but not the PMP) after MP coagulation has occurred [see Figure 3 in Minami *et al.* (2012)].

To determine whether the identified MP proteins are important for *Drosophila* fertility, we knocked down two additional abundant MP proteins in males and examined egg laying in mates of those knockdown males. We found that knocking down either CG15616 (Acp53C14a) or CG8626 (Acp53C14b) significantly impaired egg laying (Figure S8). This analysis further demonstrates the importance of the MP and the proteins that comprise it in *Drosophila* fertility.

Conclusions

We have shown the importance of PEBme, and, by extension, the PMP, in *Drosophila* reproduction. PMP formation occurs quickly in the female reproductive tract after mating begins and is retained for several hours, until the structure is expelled (Manier *et al.* 2010; Lee *et al.* 2015). When *PEBme* was knocked down in males, PMP coagulation was severely reduced or absent in their mates. This caused removal of the ejaculate from the uterus as mating pairs uncoupled. Extraction of ejaculate during uncoupling is a unique phenotype that, to our knowledge, has not been described previously in MP studies of other species.

One would expect the inability to cleanly uncouple to negatively affect fertility due to sperm and SFP loss. Indeed, we observed defects in uterine conformation, sperm storage, and egg laying in mates of *PEBme* knockdown males. This shows that PEBme (and proper PMP formation) is required to prevent ejaculate loss when males disengage from females at the end of mating. However, this effect was not absolute. We also observed some females with high levels of sperm in storage shortly after mating ended (35 min ASM), but when sperm storage was assessed at 24 hr ASM, we detected a significant reduction of sperm in storage in mates of *PEBme* knockdown males. This suggests that even when the ejaculate was maintained within some females at the termination of copulation, ejaculate loss occurred soon thereafter. Thus, an additional function of the PMP might be to prevent MP ejection after mating, providing time for sperm to be stored at maximal levels and for SFPs to exert their effects on mated females (*e.g.*, inducing ovulation and promoting sperm storage).

Our results show that MP formation and ejection are important aspects of *Drosophila* reproduction. This analysis

has begun to elucidate the male molecules required for MP formation, but female molecular contributions to MP formation and ejection are largely unknown. MP dynamics may influence postcopulatory sexual selection. In doubly -mated *Drosophila* females, the timing of MP ejection influences paternity—the later that MP ejection occurs, the more progeny are sired by a second mating male (Lüpold *et al.* 2013). Female molecular contributions to MP formation and/or ejection may be found among secreted components of the female reproductive tract (Allen and Spradling 2008; Prokupek *et al.* 2009; Schnakenberg *et al.* 2011; Wong *et al.* 2012; Sun and Spradling 2013), neuromodulators (Avila *et al.* 2012; Heifetz *et al.* 2014), and/or innervation (Middleton *et al.* 2006; Rubinstein and Wolfner 2013) required for fertility. In particular, a recent study showed that the activity of the neuropeptide diuretic hormone 44 in a subset of *doublesex*-expressing neurons is required for MP ejection (Lee *et al.* 2015). Future investigations into these female molecules and/or neural components also will greatly add to our understanding of *Drosophila* reproduction.

Acknowledgments

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GENETICS

Supporting Information
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Retention of Ejaculate by *Drosophila melanogaster* Females Requires the Male-Derived Mating Plug Protein PEBme

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DOI: 10.1534/genetics.115.176669

SUPPORTING FIGURES

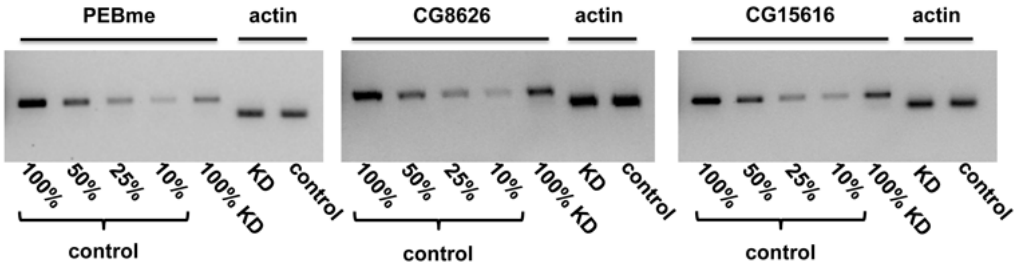

Figure S1. Knockdown of MP proteins. For each genotype, a dilution series of PCR products from the cDNA of control males was compared to the PCR product from knockdown (KD) males to obtain an estimate of the level of knockdown achieved in our experimental males. All PCR products were obtained at 30 cycles. Actin5C PCR products from both knockdown and control male cDNA were used as a control. *CrebA*-GAL4, a male ejaculatory bulb driver, was used to knockdown *PEBme*. *ovulin*-GAL4, a male accessory gland driver [27], was used to knockdown CG8626 and CG15616. We estimate the following levels of expression relative to controls: *PEBme* ~25%; *CG8626* ~50%; *CG15616* ~50%.

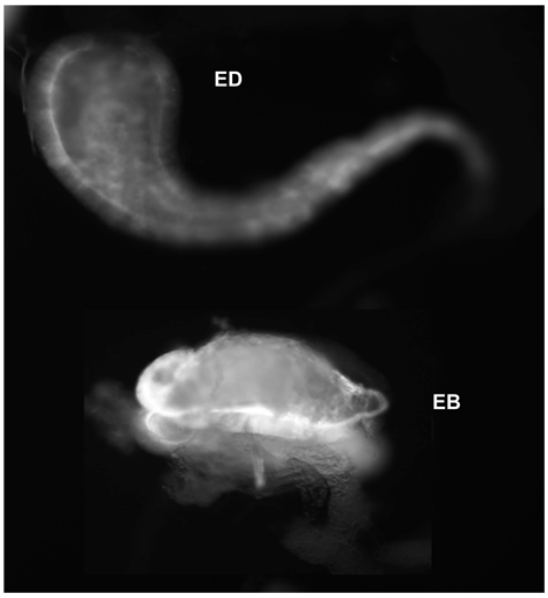

Figure S2. *CrebA*-GAL4 drives expression into the ejaculatory duct (ED) and ejaculatory bulb (EB) of the male reproductive tract. Visualization of *mCD8::GFP* driven by *CrebA*-GAL4 in the ED and EB of the male reproductive tract.

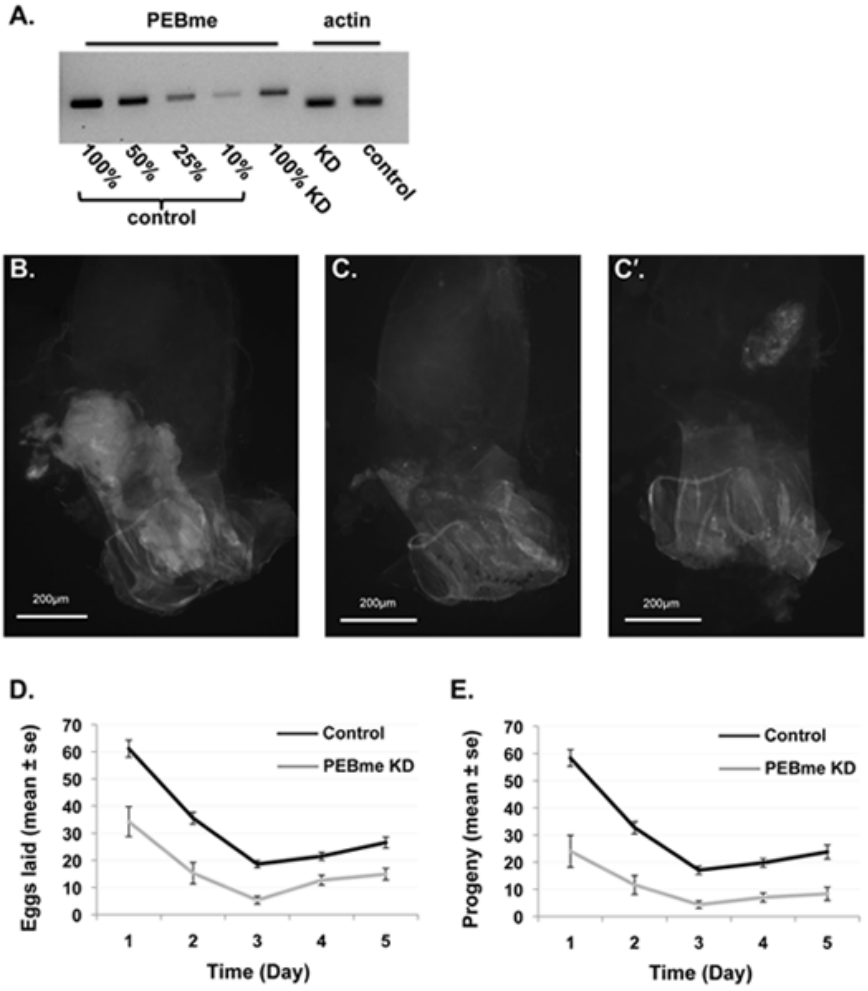

Figure S3. Knockdown of the *PEBme* using an additional dsRNA transgenic line perturbs MP formation and female egg-laying. (A) Knockdown of *PEBme* (VDRC line #18973) by *CrebA*-GAL4. We estimate *PEBme* expression of 50% relative to controls. Control males were generated by crossing the *CrebA*-GAL4 line to w^{1118} females (the VDRC line #18973 background line). Compared to mates of controls (B), mates of $PEBme^{18973}$ knockdown males have perturbed MP formation (C, C′), leading to a reduction in eggs laid (D) and progeny produced (E).

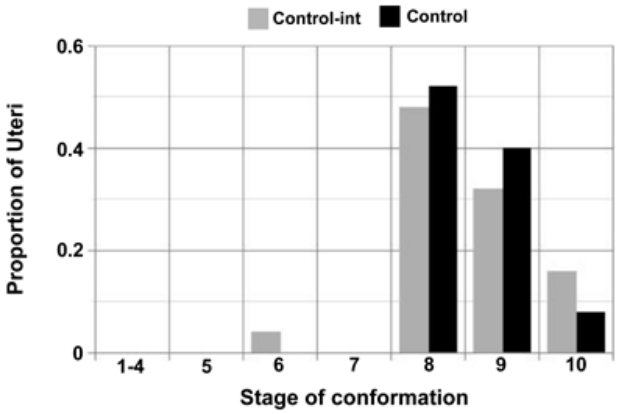

Figure S4. Interrupting mating after sperm and SFP transfer does not affect progression of the uterine conformational changes. Comparison of females mated to control males when mating was interrupted at 13min (Cont-int) vs. mating that was not interrupted (Cont). Uterine progression occurs normally once females have received SFPs during mating ($N_{cont\text{-}int}$=25, N_{cont}=25, Z=0.11, *P*=0.92).

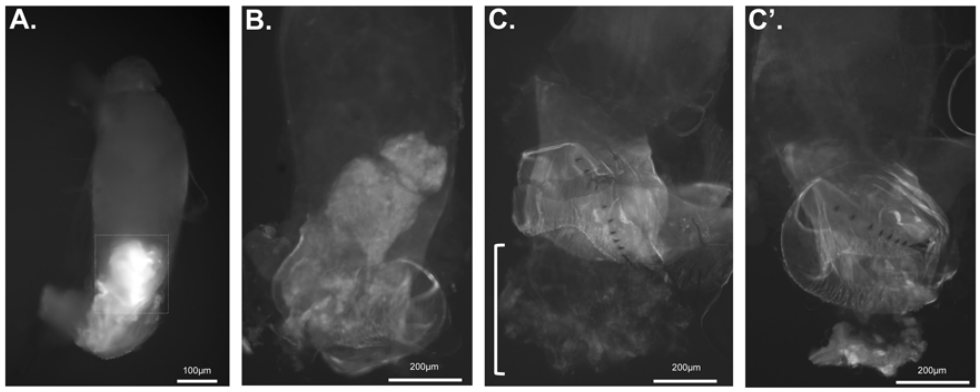

Figure S5. Blocking translation of proteins in the EB impairs PMP formation. (A, B) Mates of control males have fully formed PMPs in the posterior uterus at 35min ASM. (C, C') Mates of *CrebA>Rh1*^{G69D} males have non-coagulated PMPs (these are extruded from the uterus after application of the coverslip; bracket in C) or reduced PMPs at 35min ASM (C'). The uterus in (A) was not placed under a coverslip (the PMP is highlighted by the dotted box). To better visualize the PMP, the uteri in (B-C') were placed under a coverslip; B, C and C' are shown under the same magnification. PMPs are visualized under UV illumination.

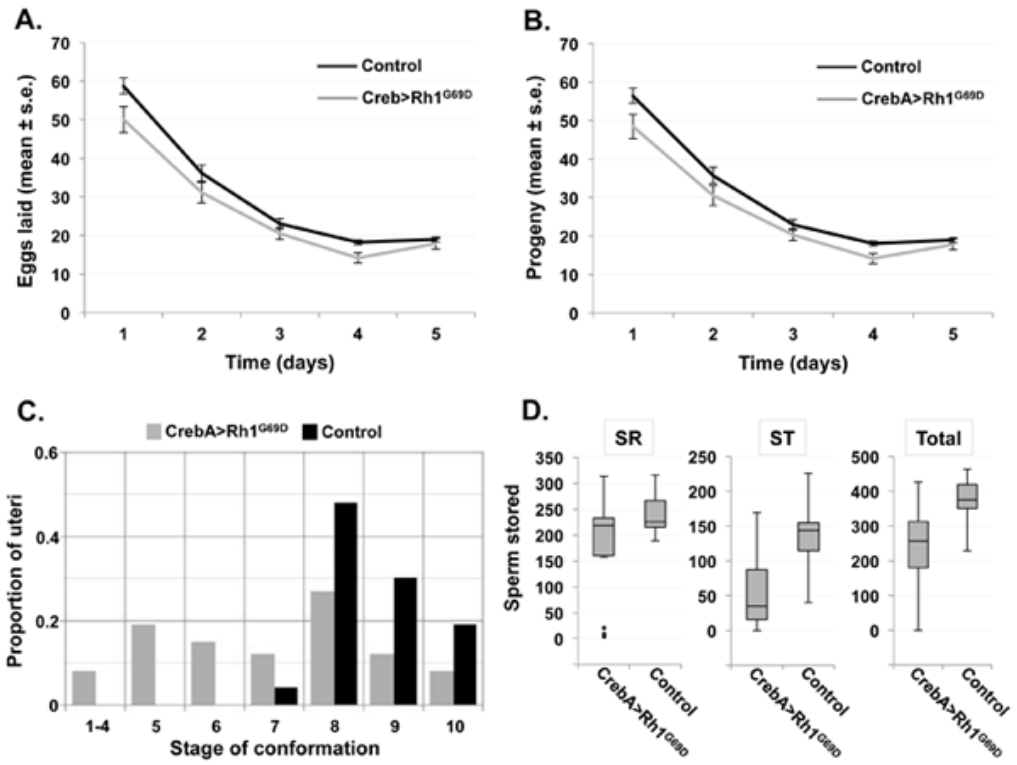

Figure S6. Mates of *CrebA>Rh1^{G69D}* males **are subfertile.** (A) Mates of *CrebA>Rh1^{G69D}* males lay significantly fewer eggs (N_{Rh1}=23, N_{Cont}=21, df=1, F=5.09, *P*=0.0293) and (B) produce significantly fewer progeny (N_{Rh1}=23, N_{Cont}=21, df=1, F=4.78, *P*=0.0345) than mates of control males over a 5d period. (C) Progression of the uterine conformational changes significantly differs from controls in mates of *CrebA> Rh1^{G69D}* males (N_{Rh1}=26, N_{cont}=27, Z=-3.56, *P*=0.0004). (D) In mates of *CrebA> Rh1^{G69D}* males, sperm in storage was significantly less than in controls at 24hrs ASM (represented as box plots; SR: N_{Rh1}=19, N_{cont}=19, Z=-2.07, *P*=0.037; ST: N_{Rh1}=18, N_{cont}=16, Z=3.17, *P*=0.0015; Total: N_{Rh1}=18, N_{cont}=16, Z=2.95, *P*=0.0032). SR: seminal receptacle; ST: spermathecae; Total: SR + ST.

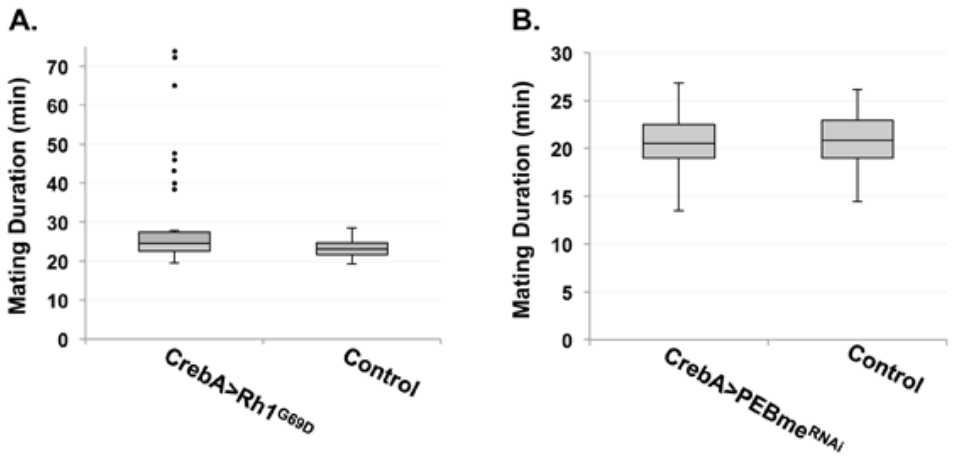

Figure S7. Mating duration of *CrebA>Rh1^{G69D}* and *CrebA>PEBme^{RNAi}* males. (A) *CrebA>Rh1^{G69D}* males mate significantly longer than their controls, with 15% of matings exceeding 38min (N_{Rh1}=50, N_{Cont}=45, $t(52)$=2.91, P=0.005). (B) *CrebA>PEBme^{RNAi}* males do not differ from their controls in mating duration (represented as box plots; N_{PEB}=45, N_{Cont}=40, $t(82)$=0.69, P=0.49).

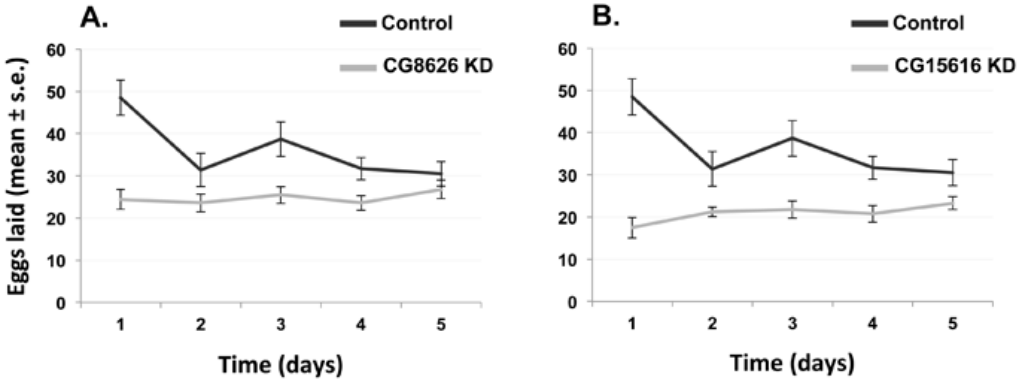

Figure S8. Knockdown of the MP proteins CG8626 or CG15616 impairs egg-laying.

(A) Mates of *ovu>CG8626*^{RNAi} and (B) *ovu>CG15616*^{RNAi} males lay significantly fewer eggs than mates of control males over the first 5d post-mating (N_{CG8626}=13, N_{Cont}=12, df=1, F=14.27, *P*=0.001; $N_{CG15616}$=13, N_{Cont}=12, df=1, F=25.48, *P*<0.0001).

Files S1-S4

Available for download as .mp4 files at

www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.176669/-/DC1

File S1. A *PEBme* knockdown male and Canton S female remain attached via the ejaculate after mating has ended.

File S2. A *PEBme* knockdown male incidentally pulls the ejaculate while uncoupling from the female. When the male successfully detaches, the ejaculate is seen protruding from the female reproductive tract.

File S3. A *PEBme* knockdown male incidentally pulls the ejaculate from a Canton S female as he uncouples at the end of copulation.

File S4. A control male uncouples cleanly from a Canton S female.

Table S1. The 25 most abundant MP proteins. MP proteins identified by mass spectrometry were ranked by calculating the mean normalized spectral abundance factor, an estimate of a given protein's relative abundance in the analyzed sample.

Rank	Protein	Molecular Function	FlyBase ID
1.	**PEBII**	**unknown**	**FBgn0011694**
2.	**CG8626**	**unknown**	**FBgn0034152**
3.	**Sfp24Bb**	**serine endopeptidase inhibitor activity**	**FBgn0259952**
4.	**Acp36DE**	**unknown**	**FBgn0011559**
5.	**Acp63F**	**unknown**	**FBgn0015585**
6.	**Sfp60F**	**unknown**	**FBgn0259968**
7.	**CG15616**	**unknown**	**FBgn0034153**
8.	**CG43147**	**unknown**	**FBgn0262623**
9.	**sex peptide**	**hormone activity**	**FBgn0003034**
10.	**Acp53Ea**	**hormone activity**	**FBgn0015584**
11.	**Met75Ca**	**unknown**	**FBgn0028416**
12.	**Met75Cb**	**unknown**	**FBgn0028415**
13.	**Sfp87B**	**unknown**	**FBgn0259975**
14.	**CG17472**	**unknown**	**FBgn0032868**
15.	**Mst57Da**	**unknown**	**FBgn0011668**
16.	**ovulin**	**hormone activity**	**FBgn0002855**
17.	**CG6555**	**unknown**	**FBgn0267327**
18.	**CG17097**	**lipase activity**	**FBgn0265264**
19.	**Sfp70A4**	**unknown**	**FBpp0289544**

20.	**Sfp65A**	**unknown**	**FBgn0259969**
21.	**Sfp65A**	**unknown**	**FBgn0259969**
22.	**Acp62F**	**serine endopeptidase**	**FBgn0020509**
23.	**CG42782**	**unknown**	**FBgn0261853**
24.	**PEBme**	**unknown**	**FBgn0004181**
25.	**Sfp26Ad**	**unknown**	**FBgn0261055**

File S5

SUPPORTING METHODS

RNA extraction and RT PCR: We extracted RNA from ~20 whole experimental and control flies using TRIzol® reagent (Invitrogen). Isolated RNA was treated with RQ1 DNase (Promega) and cDNA was synthesized with SMARTScribe Reverse Transcriptase (Clontech). PCR to amplify cDNA was run for 30 cycles using primers for the specified gene. Primers used: **PEBme 5'** GGA ATT TTC GGA CAA CAT GG, **PEBme 3'** CCT TTT ACC GAT GGC ACT GT; **CG8626 5'** ATG CCA AAA GTC GCA AGT TC, **CG8626 3'** TGA TGG GCC ACC CTA ATA AA; **CG15616 5'** ATC GTC CCG ACC GTA TAT GA, **CG15616 3'** TCG TCC AAG CAA CCT AAT CG. Actin5C primers (**5'**: AGC GCG GTT ACT CTT TCA CCA C, **3'** GTG GCC ATC TCC TGC TCA AAG T) were used in parallel to serve as a loading control.

Video imaging: We recorded mating of experimental and control males with females in semi-microvolume cuvettes (Bio-Rad) using a Canon MP-E 65mm f/2.8 1-5x macro lens (ratio 2:1) mounted on a Canon 5D mark III, stabilized on a tripod (Induro CT213 Carbon 8x) and driven by a 4 way macro focus rail (Neewer Pro). Video editing was performed with Adobe Premiere Pro.

Mating duration: Experimental and control males were individually mated to females in glass vials containing moistened filter paper. The times of mating initiation and termination were recorded. Mating duration was analyzed using t-tests in JMP 9.02.