

Don't pull the plug! the *Drosophila* mating plug preserves fertility

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Mating plugs are hardened structures—typically a coagulation of seminal fluid components—that are transferred to, or formed within, the female reproductive tract of numerous animal species (both mammals and insects). Analysis of the role(s) of the mating plug in reproduction has been conducted in a wide array of diverse species. These structures have been proposed to have a multitude of functions, which include altering female re-mating rate, acting as a barrier to re-mating and being required for sperm storage or sperm movement to occur in mated females. A recent analysis of the *Drosophila melanogaster* mating plug has shown that proper formation of the structure is required for optimal fertility in flies: the *Drosophila* mating plug is required to retain the ejaculate within the female reproductive tract once mating has terminated. Here, we discuss the possible implications of the *Drosophila* mating plug in the fertility of this species in light of these new results.

Introduction

Mating plug formation—a coagulation of seminal fluid components transferred to, or formed within, the female reproductive tract—is a phenomenon that occurs during or shortly after mating in several taxonomically diverse species.^{1,2} These structures are proposed to have numerous functions, which include preventing sperm loss,³ facilitating the storage of sperm in the mated female,⁴ decreasing or eliminating female sexual receptivity^{3,5-7} and/or acting as a physical barrier to prevent female re-mating, thereby reducing the risk of sperm competition.⁸ Evidence for the latter includes a correlation of

mating plug formation in primate species with multiple mating⁹ and the accelerated rate of evolution of a major mating plug gene in polyandrous primates relative to monandrous primates.¹⁰

However, while many studies have focused on the mating plug's role in female re-mating and in sperm storage/sperm competition parameters, recent studies have suggested that in some species, mating plugs may have additional and somewhat unexpected roles in reproductive processes. For example, in matings by mutant male mice that lack the prostate-expressed transglutaminase, “copulatory plugs” do not form, and this prevents sperm migration to the sites of fertilization.¹¹ In addition, the mouse seminal protein SVS2, which is also required for proper copulatory plug formation, is required for sperm survival in the uterus.¹² These data suggest that the copulatory plug may have a sperm protective function, as well as facilitating sperm motility, in the female mouse reproductive tract. In another example, presence of a mating plug in anopheline mosquitoes has been suggested to associate with malaria-vectorial capacity.¹³ In species where malaria transmission is high, coagulated mating plugs form, correlating with transfer of the steroid hormone 20E to females during mating. 20E interacts with a female protein that regulates expression of lipid transporters resulting in increased oogenesis and favoring development of *Plasmodium*, the malaria parasite.¹³⁻¹⁵ Examples such as these suggest that the mating plug likely functions in processes aside from the oft-cited roles of preventing re-mating and effecting sperm storage.

The *Drosophila* mating plug

Little is known about mating plug formation across *Drosophila* species.

Keywords: *Drosophila* reproduction, mating plug, PEBme, sperm storage

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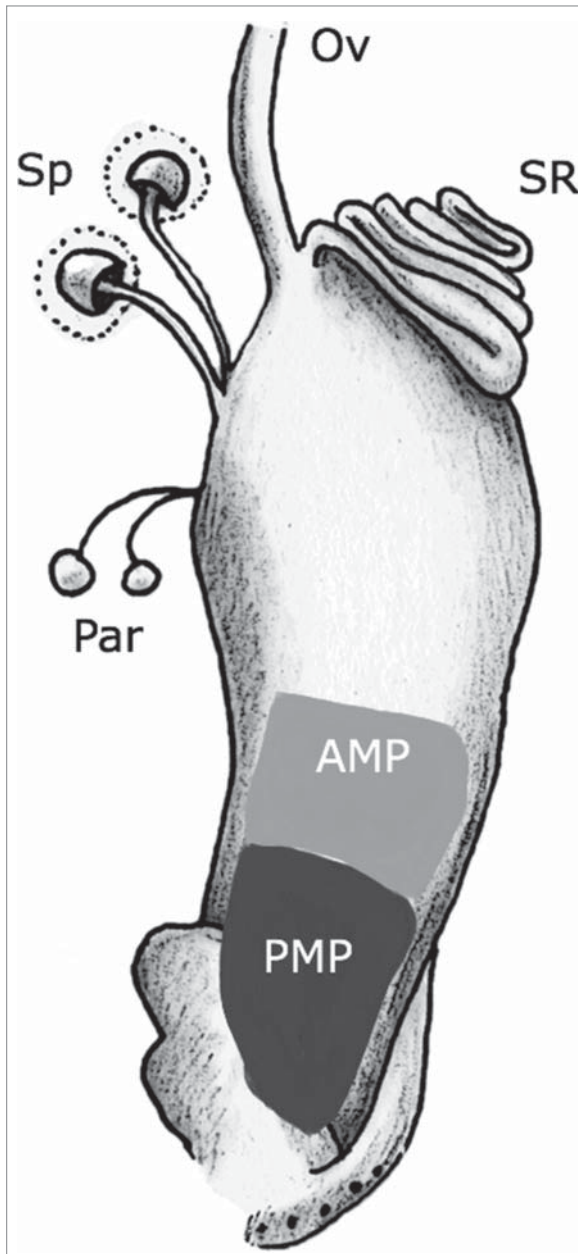


Figure 1. Diagram of the mating plug in the *Drosophila melanogaster* female lower reproductive tract. The uterus is shown containing the mating plug (AMP + PMP; for anterior + posterior mating plug, respectively). The figure also shows the sperm storage organs (seminal receptacle (SR), spermathecae (ST)), the parovaria (Pa), and the lower common oviduct (Ov). The upper reproductive tract (ovaries, lateral oviducts, upper common oviduct) is not shown, for simplicity.

Alonso-Pimental et al.¹⁶ distinguished among several types of structures formed in mated female reproductive tracts of different *Drosophila* species. For example, they reported that *D. hexastigma* has a hardened mating plug whose description seems similar to what Bairati and Perotti¹⁷ observed in *D. melanogaster*. A

reproductive tract. The rapid-forming posterior mating plug (PMP) is derived from proteins secreted from the male's ejaculatory bulb^{6,21-23} while the later-forming anterior mating plug (AMP) is derived primarily from proteins secreted from the male's accessory glands.^{23,24} Removal of the accessory gland-derived seminal protein

mating plug has also been reported in *D. hibisci*, but it differs from *D. melanogaster*'s in being a gelatinous structure that fills the lumen of the female reproductive tract and remains for several days post-mating.^{3,7} Alonso-Pimental et al. also describe structures that they call "sperm sacs" in *D. mettleri* and in *D. nigrospiracula*. They describe these structures as softer and less distending than true mating plugs. (They suggest that the *D. melanogaster* plug may fall into this category, but Bairati and Perotti's,¹⁷ and our¹⁸; see below), data favor its fitting into their "mating plug" category.) Finally, they describe the insemination reaction mass that forms after mating in the female reproductive tract of some cactophilic *Drosophila*.¹⁹ The insemination reaction mass also fills the female reproductive tract after mating and prevents egg-laying and re-mating until it is cleared by the female. Its size and persistence are heightened in interspecific and intra-specific interpopulation matings.²⁰

Drosophila melanogaster affords the possibility of taking a genetic approach to dissect mating plug function. The mating plug of *D. melanogaster* consists of 2 distinct regions (Fig. 1) that form sequentially and are composed of proteins from different tissues of the male

Acp36DE from the ejaculate prevents AMP formation²⁴ and hinders sperm storage.²⁴⁻²⁶ However, because Acp36DE localizes to the sperm mass as well as to female reproductive tract tissues,²⁴ it is not yet known whether the sperm storage defects observed in mates of Acp36DE null males are due to the lack of AMP formation or to additional functions of Acp36DE in mated females.

The role of the PMP in reproductive events was even less well understood. The rapid formation of the PMP (~5 min),²¹ its density, and its formation immediately inside the entrance to the female reproductive tract²³ made dissecting its role in reproduction of particular interest. To test the role of the PMP we initially attempted to suppress the production of all secreted proteins of the ejaculatory bulb.^{18,22} From the *Drosophila* Fly Lights GAL4 collection,^{27,28} we identified a GAL4 driver that expressed strongly in the ejaculatory bulb. With this CREB-GAL4 driver we generated male flies that express a misfolded rhodopsin in the ejaculatory bulb; this genotype induced ER stress in the ejaculatory bulb and thereby prevented production of its secreted proteins.^{18,22} Unfortunately, CREB-GAL4; UAS-Rh1^{G69D} transgenic males had behavioral abnormalities, likely due to expression of the driver (and hence induction of ER stress) outside the ejaculatory bulb, that made their reproductive phenotype difficult to interpret. Functional tests of the role of the ejaculatory bulb thus had to focus on specific ejaculatory bulb proteins. Proteomic analysis of the *D. melanogaster* mating plug identified potential candidates that could be targeted for RNAi knockdown.¹⁸ The first ejaculatory bulb/PMP protein targeted for RNAi-mediated knockdown was Protein of the Ejaculatory Bulb II (PEBII).⁶ Removal of PEBII from the ejaculate (via ubiquitous RNAi knockdown) reduced mating plug size and affected the short-term sexual receptivity of recently mated females but had no observable effect on female fertility.⁶

Dissecting *D. melanogaster* mating plug function

To attempt to get a broad view of ejaculatory bulb function, we decided to study

the Protein of the Ejaculatory Bulb in *melanogaster* (PEBme).²⁹ PEBme, a UV auto-fluorescent protein,²¹ has sequence features consistent with a structural role, including repetitive PGG motifs similar to those found in homopolymer-forming proteins.²¹ Putative orthologs of PEBme, defined here as reciprocal best BLAST hits, are detectable throughout the subgenus *Sophophora*. Orthologs are not detectable in the subgenus *Drosophila*, including the desert *Drosophila*, or in other dipterans. PEBme has well-conserved N- and C-terminal regions, with a highly variable central region spanning amino acids 127–273 of the 377 aa of the *D. melanogaster* protein (Fig. 2). This variable region is highly repetitive, and is particularly rich in G, L, P and S residues. Alignments among putative PEBme orthologs from *D. melanogaster*, *D. simulans*, *D. erecta*, *D. yakuba*, *D. ananassae*, and *D. elegans* suggest a high frequency of insertions and deletions in this central region. This high sequence variability could in principle result from diversifying selection, perhaps due to effects of PEBme in sperm competition or sexual conflict.³⁰ Alternatively, if the central region of PEBme is needed to function in a largely structural role, there may be low levels of constraint on its sequence; perhaps any (or various) repeated homopolymer-generating sequence will do, allowing for high levels of neutral divergence. Unfortunately, the repetitive and simple-sequence nature of the central region of PEBme makes sequence alignments highly gapped and uncertain, preventing standard tests for positive selection based on between-species divergence, such as those implemented in PAML.³¹ Alternative, population-based approaches may instead allow for formal neutrality tests if alignments are more trustworthy.

Due to its potential structural role, we reasoned that knockdown of PEBme might disrupt mating plug formation. PEBme's role in mating plug formation and fertility were unknown and null mutations of the gene are unavailable. We attempted to knock down *PEBme* ubiquitously, but this resulted in late-pupal lethality. This lethality may reflect a requirement for *PEBme* expression in other tissues (i.e. the digestive and/or respiratory system, where *PEBme* is also expressed),³²

or could be due to a non-specific, dominant effect for this particular line, similar to effects reported for a subset of KK RNAi lines when crossed to a GAL4 driver.³³

To circumvent the lethality of ubiquitous *PEBme* knock-down, we used the CREB-GAL4 driver to target *PEBme* for RNAi knockdown in (or primarily in) the ejaculatory bulb.¹⁸

Knocking down PEBme to ~25% of wild-type levels was sufficient to nearly eliminate mating plug formation and had drastic consequences on female fertility.¹⁸ When mating plug formation was perturbed or prevented in this way, mated females were significantly compromised in their ability to increase egg production and to store sperm after mating.¹⁸ Moreover these females did not properly undergo the changes in uterine conformation¹⁸ that normally occur after mating and are essential for sperm storage.^{25,34} The breadth of phenotypes suggested that the reduction in fertility in these females was likely due to a phenomenon that affected the entire ejaculate, such as loss of ejaculate in females mated to *PEBme* knockdown males. Indeed, in the absence of PMP formation, the ejaculate was often not maintained in the female reproductive tract. Rather, it failed to coagulate properly and thus was incidentally pulled from the female when males uncoupled. These results suggested that coagulation of the PMP may allow the males to disengage cleanly once mating has ended. Thus, our experiments showed that in *D. melanogaster*, mating plug formation is crucial for fertility and for aspects of mating itself.

At first glance, these results support previous reports about the requirement of a mating plug to prevent sperm “leakage” or “backflow” from the female reproductive tract.³ However, careful consideration of the observed phenotypes suggests that mating plug formation may support

fertility in numerous additional ways, some of which are analogous to mating plug functions in other organisms.

Potential roles of the *D. melanogaster* mating plug in mating and fertility

The most notable phenotype that we observed in mates of *PEBme* knockdown males was complete loss of the ejaculate from the reproductive tracts of mated females.¹⁸ This phenotype was significantly exacerbated when the mating animals were shaken lightly. This level of agitation, sufficient to prematurely terminate matings by knockdown and control males, did not impact the fertility of control males, but greatly exacerbated the fertility defects of knockdown males. These results suggest that the PMP may support copulating males by “holding” them in place for the duration of copulation. If this hypothesis is correct, it suggests that mutations that perturb mating plug formation may have drastic consequences in wild *D. melanogaster* populations. In the wild, mating often occurs in proximity to rotting fruit, which attracts large numbers of flies³⁵ including competitor males that are likely to jostle copulating pairs. Moreover, under such crowded conditions, the threat of sperm competition would be high—a situation in which sperm and/or seminal fluid transfer may be increased.^{36,37} In such a situation the

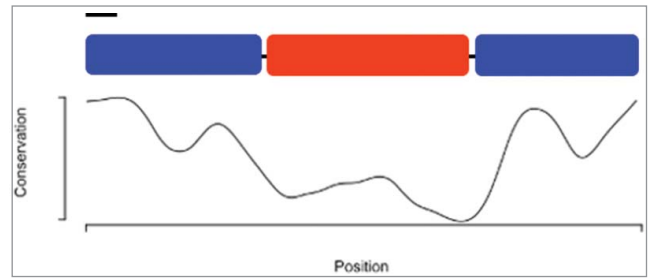


Figure 2. A schematic of the PEBme open reading frame, above a graph of amino acid sequence conservation. The protein is drawn to-scale, with the black bar indicating the predicted signal sequence. PEBme's ends (blue) are more highly conserved in sequence than its central region (red), which contains the repeated motifs. Conservation scores were annotated using the physico-chemical scores of Livingstone et al.,⁵³ as implemented in JalView.⁵⁴ The graph was generated in R.⁵⁵ Scores range from 0 (no conservation) to 11 (full conservation among all species).

male benefits of increased ejaculate allocation would still require maintenance of the ejaculate in the female reproductive tract and therefore necessitate proper mating plug formation.

Mating plugs have been hypothesized to act as a form of mate guarding. In *D. melanogaster*, the mating plug does not prevent sperm transfer from subsequent competitor males—sperm from both males can be observed in the few females that re-mate shortly after an initial mating.³⁸ Further, females that mate to males lacking sex peptide (a seminal protein that suppresses female re-mating¹) will often quickly re-mate with a second male (FWA, unpublished observations) even though these females have fully formed PMPs.²³ Thus, mating-induced refractoriness to re-mating resulting from seminal protein receipt and the formation of the mating plug may simply ensure the storage and subsequent usage of an initial male's sperm.

Upon mating, sperm storage in the female reproductive tract occurs rapidly, beginning ~25 min after copulation begins.³⁸ Females ultimately expel the remainder of the ejaculate (mating plug plus non-stored sperm) several hours after mating ends.^{38,39} The timing of ejaculate expulsion affects sperm competitive success in multiply-mated females.⁴⁰ Given the phenotypes of *PEBme* knockdown males, we suggest that PMP coagulation may provide a mechanism to delay ejaculate ejection, ensuring that the males' sperm are stored in maximal numbers and providing sufficient time for seminal fluid proteins to elicit their effects (more sperm in storage results in more sequestered sex-peptide in the female reproductive tract⁴¹ which will prolong female remating-refractoriness). In view of potential conflicts between the reproductive strategies and interests of males and females, it will be interesting to investigate whether some proteases secreted by the female reproductive tract⁴²⁻⁴⁴ might work to degrade the mating plug allowing it to be expelled more quickly.

In addition to keeping the ejaculate contained within the female reproductive tract, it is likely that the PMP, or the MP as a whole, acts as a scaffold to support sperm storage and, possibly, seminal

protein function. The PMP forms quickly, ~5min after mating begins²¹ and is followed by the 'opening' of the uterus,^{25,34} formation of the AMP,²⁴ the organization and movement of sperm toward the female sperm storage organs³⁴ and, finally, active sperm storage.^{25,34} PMP formation may be the first step in supporting male-derived action in the female reproductive tract by providing a structure to support subsequent AMP formation. In addition it is intriguing to speculate that the MP might facilitate sperm storage, for example by providing a scaffold, structure, or meshwork for sperm to move against, or to 'disentangle' individual sperm from the sperm mass. However, the effects of the mating plug on the characteristics and rate of sperm entry into storage are as yet unknown and require future exploration, for example in *PEBme* knockdown situations.

The mating plug may play an additional structural role. Once the ejaculate has been transferred to the female, the uterus undergoes a series of conformational changes that depend on the receipt of seminal proteins from the male's accessory gland.^{25,34} However, in the absence of transfer of accessory gland proteins, the posterior mating plug still forms and causes an expansion in volume of the posterior uterus.^{22,23} It has been proposed that sensory neurons of the *Drosophila* uterus are mechanosensory and therefore act as stretch receptors, sensing that mating has occurred and coordinating post-mating processes such as fertilization and egg-laying.⁴⁵⁻⁴⁷ The volume and solidification of the mating plug may contribute to distending the uterus, thereby activating the stretch receptors to signal an initiation of female post-mating responses.

In addition to secreting PMP proteins, the ejaculatory bulb is also the site of synthesis of male pheromones, including triacylglycerides⁴⁸ and *cis*-vaccenyl acetate (cVA).⁴⁹ These molecules are transferred to the female reproductive tract during mating.^{48,50} While triacylglycerides are not expressed sex-specifically in *D. melanogaster*, cVA is part of the hydrocarbon profile of *Drosophila* males and influences courtship and aggression behaviors.^{50,51} It is possible that mating plug formation supports

the positioning or concentration of pheromones at the appropriate place in the female reproductive tract.

Conclusion

Mating plugs actively contribute to fertility. In addition to the well-defined roles of mating plugs in supporting sperm storage and affecting sexual receptivity, the phenotypes of *PEBme* knockdown males showed that the *Drosophila melanogaster* mating plug is required for the maintenance of the ejaculate within the post-mated female reproductive tract, and for allowing males to uncouple cleanly after the termination of copulation. However, many fascinating questions about mating plugs remain to be addressed. For instance, now that constituents of the *D. melanogaster* mating plug, and some of their functions, are known it will be interesting to compare the biochemistry of such post-mating structures across *Drosophila* species, including whether and how *PEBme*-like proteins are involved in the formation of other mating plug-like structures, such as the insemination reaction masses that form in heterospecific matings among desert *Drosophila* species.⁵² These questions are particularly interesting given the diversity of such post-mating structures even within species (among populations)²⁰ as well as between species. Relatedly, the roles of the many other proteins identified by proteomic analysis of the mating plug will be interesting to discover, as these are still unknown even in *D. melanogaster*.¹⁸ It will also be interesting to determine the extent of variation in mating plug coagulation in wild *Drosophila melanogaster* (or across laboratory strains), and whether and how this affects fertility. Another interesting set of questions relates to the likely role of mating plugs in sexual conflict as well as in fertility.²⁰ In this context, a detailed evolutionary analysis of *PEBme* may identify whether its putative structure-generating region is under positive selection, or under relaxed selection, and may pinpoint features that play a structural role. Finally, what role does the mating plug play in sperm movement in the female reproductive tract, and does

mating plug formation activate reproductive tract stretch receptors? The multitude of molecular and genetic tools available for use in *Drosophila* will allow researchers to address these, and other, pressing questions about mating plug function and contents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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