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A mating plug protein reduces early female remating in *Drosophila melanogaster*

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ABSTRACT

Mating plugs are formed within the female reproductive tract during mating from male ejaculate constituents or even from male genitalia themselves. Across species, mating plugs have roles in sperm storage and the prevention of female remating. In the fruitfly *Drosophila melanogaster*, accessory gland proteins such as the sex peptide are known to reduce female remating, however this effect can take some time to establish, hence other ejaculate components must also be involved. We hypothesised a role for the PEBII mating plug protein in the prevention of early female remating. Using RNA interference we produced PEBII knockdown males. We found that these males were significantly less able to prevent female remating in the 4 h following mating. The mating plugs produced by PEBII knockdown males also showed lower levels of autofluorescence in the first 10 min after the start of mating, suggesting they differed in composition to those of control males. Reduced levels of PEBII had no effect, however, on fecundity, progeny production or egg–adult viability in the first 24 after mating, suggesting there were no short-term effects of PEB II on sperm transfer, storage or use. Our results show that PEBII has a subtle but significant role in the prevention of early female remating.

1. Introduction

In many species, a mating plug is formed within the reproductive tract during and/or after mating. Such plugs have been shown to have a range of functions related to sperm competition (e.g. Mann, 1984; Eberhard, 1996; Birkhead and Møller, 1998; Simmons, 2001). Most obviously, plugs form a physical barrier to prevent remating or to prevent loss of sperm female reproductive tract. In butterflies, plugs (referred to as sphraga) physically prevent remating and are also large and elaborate, perhaps to act as visual deterrents to other males (Orr and Rutowski, 1991). Plugs can reduce female receptivity in the short term (especially if they must be expelled to allow oviposition), or in extreme cases such as the bumblebee *Bombus terrestris*, the plug permanently switches off female receptivity (Baer et al., 2001). The constituents of mating plugs vary, and can comprise proteins or lipids (Mann, 1984), but plugs can also be formed from part of the male's genitalia, the formation of which irreversibly damages the male intromittent organ (e.g. in spiders Fromhage and Schneider, 2006) or even results in male death (e.g. stingless bees Colonello and Hartfelder, 2005). The mating plug can also signal the dominance of the previous male to further prospective mates, as for example in the Iberian rock lizard *Lacerta monticola* (Moreira et al., 2006). In some cases the mating plug is also essential for females to stimulate oviposition (Melo et al., 2001).

It has been suggested that the mating plug in *Drosophila* may not prevent remating, as females remain unreceptive until after plug is degraded (Eberhard, 1996). However, this overlooks the possibility that females are unreceptive because of chemical constituents of the plug, and this is the topic we investigate in this study. Recent evidence suggests that the mating plug in *Drosophila* reduces female remating and facilitates sperm storage. Experimental reduction of the mating plug size through manipulating mating duration in *Drosophila hibisci*, for example, demonstrates how the plug reduces female remating by inhibiting courtship by other males and by reducing female receptivity (Polak et al., 2001). Polak et al. (1998) also showed that the plug ensures sperm storage; smaller plugs from previously mated males could not prevent back-flow of sperm away from the sperm storage organs or out of the female.

In *Drosophila melanogaster*, the mating plug comprises two major portions; the posterior portion contains proteins synthesised in the male ejaculatory bulb (PEB-me, PEBII and PEBIII), and proteins from the male accessory gland (including Acp36DE) are contained within the anterior plug (Lung and Wolfner, 2001). The posterior plug is formed within 3 min after the start of mating (ASM) (Lung and Wolfner, 2001) but before sperm transfer

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(Gilchrist and Partridge, 2000), hence the sperm may permeate or move around the plug before the anterior plug is formed at 20 min ASM. Males lacking Acp36DE (the anterior plug) transfer normal amounts of sperm, but fewer of their sperm are stored and females mated to these males consequently have fewer offspring (Neubaum and Wolfner, 1999).

We investigated here the effect of one of the constituent proteins of the *D. melanogaster* mating plug, PEBII, on female receptivity immediately after the first mating. PEBII was singled out for testing because of its potential involvement in sexual conflict between the evolutionary interests of males and females. Association studies have linked variation at the PEBII locus to increased female death rate following single matings (Fiumera et al., 2006). Hence PEBII is predicted to be subject to selection arising from sexual conflict because its transfer and recruitment to the mating plug presumably increases male fitness, but seems to have a negative side effect on the fitness of the females with which the male mates. Given the reported effect of mating plugs on female receptivity in other *Drosophila* species (Polak et al., 2001) and the potential involvement of PEBII sequence variation in determining mating costs (Fiumera et al., 2006), we predicted that loss of PEBII would cause females to remate more quickly. We focused on the early remating phenotype for two reasons. First, because there were no reported associations between PEBII variation and female refractoriness when tested at longer time points such as 48 h after mating (Fiumera et al., 2005), suggesting that any receptivity effect would be seen at earlier time points. Second, we had noticed anecdotally from observations of mixed sex groups containing PEBII knockdown males, that females often remated again almost immediately (MKNL, unpublished observations).

We tested the prediction that PEBII plays a role in early female remating by developing lines of flies in which PEBII was knocked down using RNA interference, so the effect of PEBII could be examined directly. The speed with which females remated immediately after having mated to control (producing full mating plugs) or knock down (producing plugs with reduced levels of PEBII) males was then measured in two independent lines of PEBII knockdown and control males. We also characterised the degree of autofluorescence of the mating plugs formed in females mated to one of the PEBII knockdown lines at 5–10 and 20 min ASM to determine whether there were gross changes in the structure of mating plugs with reduced PEBII. To examine whether any effects on early remating were associated with fecundity or fertility differences, we then conducted a third experiment in which females were mated singly to PEBII reduced or control males.

2. Materials and methods

2.1. Fly rearing and food media

Fly rearing and all experiments were conducted in a 25 °C humidified room, with a 12:12 h light:dark cycle, on standard sugar-brewer's yeast-agar (SY) medium (Bass et al., 2007) supplemented with live yeast granules. Wild-type flies were from a large, outbred laboratory population originally collected in Dahomey (Benin) (Bass et al., 2007).

2.2. Generation of PEBII knockdown lines

PEBII knockdown males were generated by RNAi using the UAS/Gal4 system, as described in Chapman et al. (2003). Briefly PEBII knockdown males were produced by cloning tail-to-tail inverted repeats of a 763 bp sequence including the entire PEBII gene into the pWIZ vector (Lee and Carthew, 2003). The structure of this vector, named pPEBII-IR, was verified by multiple restriction digestions. Transgenic flies were then constructed using standard methods as described in Chapman et al. (2003) by injection of pPEBII-IR into a w^1 genetic background. Homozygous viable and fertile stocks were obtained by backcrossing *white*$^+$ individuals to the w^1 injection stock and then crossing *white*$^+$ individuals *inter se*. Thus the genetic background of the lines remained that of the injection stock.

Two independent and stable homozygous lines were obtained, PEBII 13-7 and PEBII 8-1. PEBII knockdown males were derived by crossing males from PEBII 13-7 and PEBII 8-1 stocks to virgin females from a ubiquitous driver line (*Actin Gal4/CyO* in the w^{1118} background, stock 4414 from the Bloomington Stock Centre). The efficiency of knockdown was confirmed by using quantitative PCR with SYBR green probes and standard procedures (as in Chapman et al., 2003). qPCR confirmed that significant knockdown was achieved through RNAi, however, there was a difference between the two lines in the efficiency of the knockdown; there was a 16-fold and 2-fold reduction of PEBII in lines 13-7 and 8-1, respectively.

2.3. Effect of PEBII transfer on immediate remating

The effect of PEBII on female remating was tested by first mating wild-type females to experimental males (knockdown or control) and then challenging them immediately with wild-type males. We used both of the PEBII knockdown lines obtained to test for repeatability, but also for dosage effects, as we knew that line 13-7 had a much more effective knockdown than line 8-1. Males from each knockdown line were mated with virgin females from the *Actin Gal4/CyO* driver line. Hence two types of control were necessary, to control for the chromosomal location of the *Gal4* driver and for the location of each of the 2 PEBII inverted repeat constructs. We also equalised as far as possible the degree of red eye-colour in the controls in comparison to the experimental lines. This was necessary because the degree of orange pigment in the eyes (as determined by the copy number of transgenic inserts marked with w^+) affects male mating success (AB and TC unpublished data) presumably because *white*-eyed males are visually impaired. Therefore, we controlled for this by creating control and experimental lines all with 2 transgene copies. To do this we crossed the *Actin Gal4/CyO* driver and PEBII inverted repeat lines separately to a transgenic line that contained a non-functional *UAS-Sex Peptide* construct (*UAS-SP-stop*, marked with w^+) in the same w^1 background as used for the PEBII inverted repeat lines. Specifically, knockdown controls were created by crossing PEBII 13-7 and 8-1 knockdown line males to virgin females from the *UAS-SP-stop* line, and the driver controls from crossing *UAS-SP-stop* males with *Actin Gal4* driver virgin females. The non-functional *UAS-SP-stop* construct has no detectable effects on any other mating traits tested so far and is simply used here to control for eye-colour.

Larvae from these crosses and from the Dahomey population were maintained at a standard density of 100 per vial. At eclosion offspring were collected using ice anaesthesia and separated into single sex vials at 10 per vial. At 4 days post-eclosion males were transferred singly into experimental vials using ice anaesthesia. The following day females were aspirated singly into the mating vials. Introduction time, copulation start and end time were noted, with great care taken not to disturb the vials (as this is known to significantly shorten mating duration, AB and TC unpublished data). Flies that did not mate within 1 h were discarded. After mating had ended the female was aspirated into a vial containing a wild-type male and again the introduction, start and end of mating times were noted. The transfer time between the end of the first