The Acp26Aa seminal fluid protein is a modulator of early egg hatchability in *Drosophila melanogaster*

Tracey Chapman^{1*}, Laura A. Herndon^{2†}, Yael Heifetz², Linda Partridge¹ and Mariana F. Wolfner²

¹The Galton Laboratory, Department of Biology, University College London, Wolfson House, 4 Stephenson Way, London NW1 2HE, UK

²Department of Molecular Biology and Genetics, Biotechnology Building, Cornell University, Ithaca, NY 14853-2703, USA

Drosophila melanogaster male accessory gland proteins (Acps) that are transferred in the ejaculate with sperm mediate post-mating competition for fertilizations between males. The actions of Acps include effects on oviposition and ovulation, receptivity and sperm storage. Two Acps that modulate egg production are Acp26Aa (ovulin) and Acp70A (the sex peptide). Acp26Aa acts specifically on the process of ovulation (the release of mature eggs from the ovaries), which is initiated 1.5 h after mating. In contrast, sperm storage can take as long as 6–9 h to complete. Initial ovulations after matings by virgin females will therefore occur before all sperm are fully stored and the extra eggs initially laid as a result of Acp26Aa transfer are expected to be inefficiently fertilized. Acp26Aa-mediated release of existing eggs should not cause a significant energetic cost or lead to a decrease in female lifespan assuming, as seems likely, that the energetic cost of egg laying comes from *de novo* egg synthesis (oogenesis) rather than from ovulation. We tested these predictions using *Acp26Aa*¹ mutant males that lack Acp26Aa but are normal for other Acps and *Acp26Aa*² males that transfer a truncated but fully functional Acp26Aa protein. Females mating with *Acp26Aa*² (truncation) males that received functional Acp26Aa produced significantly more eggs following their first matings than did mates of *Acp26Aa*¹ (null) males. However, as predicted above, these extra eggs, which were laid as a result of Acp26Aa transfer to virgin females, showed significantly lower egg hatchability. Control experiments indicated that this lower hatchability was due to lower rates of fertilization at early post-mating times. There was no drop in egg hatchability in subsequent non-virgin matings. In addition, as predicted above, females that did or did not receive Acp26Aa did not differ in survival, lifetime fecundity or lifetime progeny, indicating that Acp26Aa transfer does not represent a significant energetic cost for females and does not contribute to the survival cost of mating. Acp26Aa appears to remove a block to oogenesis by causing the clearing out of existing mature eggs and, thus, indirectly allowing oogenesis to be initiated immediately after mating. The results show that subtle processes coordinate the stimulation of egg production and sperm storage in mating pairs.

Keywords: *Drosophila melanogaster*; reproductive success; Acp26Aa; seminal fluid; egg production; fertilization

1. INTRODUCTION

In species such as *Drosophila melanogaster*, in which females mate multiple times and retain the sperm of more than one male in storage (e.g. Harshman & Clark 1998; Imhof *et al.* 1998; Civetta 1999; Price *et al.* 1999), mating does not guarantee a male's reproductive success (Parker 1970). Males whose sperm are efficiently stored, whose ejaculates displace any sperm already in storage and whose sperm are efficiently used will be at an advantage (e.g. Simmons & Siva-Jothy 1998). In addition, traits that enable males to stimulate the egg production of their mates and prevent females from remating quickly will also be selected (e.g. Eberhard 1996). These post-mating effects on sperm storage, egg production and receptivity are caused in part by the actions of accessory gland proteins (Acps) that are transferred in the ejaculate together with sperm during mating (reviewed in Wolfner 1997). Thus, the Acps made in the male accessory gland main cells act to increase

male fitness. However, they can reduce female longevity and reproductive success when delivered repeatedly, thereby contributing to the 'cost of mating' (Chapman *et al.* 1995).

It is estimated that between 25 and 100 proteins are made in the main cells of the accessory gland of *D. melanogaster* (Stumm-Zollinger & Chen 1985; Wolfner 1997; Wolfner *et al.* 1997) and 18 genes encoding Acps have so far been reported (Schäfer 1986; DiBenedetto *et al.* 1987; Chen *et al.* 1988; Monsma & Wolfner 1988; Wolfner *et al.* 1997; Swanson *et al.* 2001). The functions of only a few Acps are known. Acp26Aa (ovulin) and Acp70A (sex peptide) both stimulate the egg-laying process (see below) (Chen *et al.* 1988; Herndon & Wolfner 1995; Heifetz *et al.* 2000) and both have intriguing sequence similarities to peptide hormones or their precursors (to the egg-laying hormones of the sea slug *Aplysia* (Monsma & Wolfner 1988; Heifetz *et al.* 2000) and to locust adipokinetic hormone (Chen *et al.* 1988), respectively), thereby suggesting that they too act as hormones. Acp36DE is needed for sperm storage (Neubaum & Wolfner 1999). Two Acps (Acp62F and Acp76A) have sequence characteristics of protease inhibitors (Wolfner *et al.* 1997; Lung *et al.* 2001), suggesting that they may play

*Author for correspondence (t.chapman@ucl.ac.uk).

†Present address: Molecular Biology and Biochemistry, A220 Nelson Laboratories, Rutgers University, 604 Allison Road, Piscataway, NJ 08854, USA.

a regulatory role in the action of other Acps. Some Acps show evidence of rapid evolutionary change (Aguadé *et al.* 1992; Tsaur & Wu 1997; Aguadé 1998, 2000; Tsaur *et al.* 1998; Swanson *et al.* 2001). However, the nature of the forces that drive the evolution of Acps are unknown.

In this study, we focus on an important component of a male's fertilization success, namely his ability to stimulate egg production and egg laying by his mate. The magnitude by which a male can do this will contribute directly to the number of fertilizations he achieves, provided that his sperm are used efficiently. Thus, the coordination and timing of egg production and sperm storage following mating are important components of male reproductive success. The production and laying of eggs are modulated by seminal fluid proteins (Acps) and sperm (Manning 1962, 1967; Hihara 1981; Kalb *et al.* 1993; Xue & Noll 2000). The transfer of Acp26Aa to virgin females increases ovulation and egg laying on the first day post-mating (Herndon & Wolfner 1995; Heifetz *et al.* 2000). Acp26Aa acts specifically to increase the rate of release of existing eggs (ovulation) by the ovaries (Heifetz *et al.* 2000). An examination of the effects of single matings by *Acp26Aa¹* (null) or *Acp26Aa⁺* (control) males showed that the effect of Acp26Aa on egg production is short lived (1 day) and no differences were found in egg production on subsequent days. There were also no differences in the total progeny numbers produced from a single mating by *Acp26Aa¹* or *Acp26Aa⁺* males and no differences in the receptivity of females mated to these males (Herndon & Wolfner 1995).

The injection of physiological amounts of Acp70A (Chen *et al.* 1988; Soller *et al.* 1997, 1999) stimulates the egg production of virgins for 1–2 days to a level comparable with that of mated females (Chen *et al.* 1988). Ectopic expression of Acp70A in transgenic virgin females also mimics the effect of mating on egg production (Aigaki *et al.* 1991). In addition, Acp70A can stimulate juvenile hormone BIII production *in vitro* (Moshitzky *et al.* 1996). Acp70A has been proposed to stimulate oogenesis by increasing juvenile hormone levels, since juvenile hormone induces the uptake and synthesis of yolk proteins by the ovary (Soller *et al.* 1997, 1999). Taken together, these data suggest that Acp70A causes a short-term stimulation of egg production after mating, but via a different step from Acp26Aa (oogenesis versus ovulation). The effects of both Acps are most obvious in virgin females; their effects are not easily detected against a background of high egg production in previously mated females (this study; T. Chapman, unpublished data).

Here we investigate the magnitude and timing of the Acp26Aa-mediated effect on ovulation in determining male and female reproductive success. The release of mature oocytes from the base of the ovaries, which is stimulated by the transfer of Acp26Aa, is apparent from 1.5 h post-mating (Heifetz *et al.* 2000). In contrast, sperm storage can take as long as 6–9 h after mating to complete (Gilbert 1981; Gromko *et al.* 1984; Tram & Wolfner 1999). This suggests that a male can stimulate the release of mature eggs by his mate before his sperm are completely stored. This suggestion predicts that, in first matings by females, the extra eggs produced as a result of Acp26Aa transfer will be inefficiently fertilized. This effect should only be apparent in virgin females because once the block

to ovulation is removed, oogenesis, ovulation and sperm storage and use can proceed in a coordinated manner. Acps other than Acp26Aa regulate oogenesis and sperm storage/use. Therefore, these processes are 'switched on' and coordinated in mated females regardless of whether an initial burst of ovulation occurred.

The production of eggs caused by mating may represent an energetic cost to females (Partridge *et al.* 1987; Sgrò & Partridge 1999). However, the release of existing mature eggs that is stimulated by Acp26Aa after a first mating should not contribute to this cost. The reason for this is that, although the initial energetic investment by females in the formation of those eggs (oogenesis) is expected to be significant, it is a cost incurred irrespective of the effects of Acp26Aa. Therefore, we expect the transfer of Acp26Aa *per se* to result in negligible energetic costs for females, which leads to our second prediction: that the transfer of Acp26Aa will not lead to a decrease in female lifespan or lifetime reproductive success.

The aim of this study was to test these two predictions using mutant males that lacked the Acp26Aa protein. To test whether the eggs initially produced after a first mating were fertilized less efficiently than at later times when sperm were stably stored, we examined egg fertilization rates at selected post-mating times. To determine whether changes in egg hatchability were the result of Acp26Aa transfer during first matings, virgin females were housed continuously with males that transferred no Acp26Aa (*Acp26Aa¹* males or null males) or control males that transferred truncated Acp26Aa (*Acp26Aa²* males or truncation males). We then recorded the egg and progeny production and egg hatchability of these females over their whole of their lifespans. By following females throughout their lives, we were able to test whether any effect on early egg hatchability decreased with time. *Acp26Aa²* males produce a truncated Acp26Aa protein that is fully functional, behaving like the wild-type protein in egg-laying stimulation assays (Herndon & Wolfner 1995). *Acp26Aa²* (truncation) males were derived from the same base stock and resulted from an identical mutagenesis procedure to that used to produce the *Acp26Aa¹* (null) males. In addition, *Acp26Aa²* (truncation) and *Acp26Aa¹* (null) males were each back crossed every generation to females from the same deficiency stock. Therefore, *Acp26Aa²* (truncation) males served as appropriate controls because their genetic background was extremely similar to that of the experimental males. To test the effect of Acp26Aa transfer on female longevity, we scored female survival each day until all females were dead.

Our results showed that, as predicted by the hypotheses proposed above, the extra eggs that are initially produced after a first mating are unfertilized and lower initial egg hatchability is a result of Acp26Aa transfer from first matings. Although mates of *Acp26Aa²* (truncation) males laid significantly more early eggs than mates of *Acp26Aa¹* (null) males, the number of progeny emerging from those eggs did not differ significantly between the two groups. Egg hatchability was therefore significantly lower for mates of *Acp26Aa²* males than for *Acp26Aa¹* males. Our results also showed that receipt of the Acp26Aa protein did not result in a survival cost of mating in females. We conclude that the action of Acp26Aa in releasing mature

oocytes from the ovaries is not an energetically costly process. Our results show that subtle mechanisms are at work in coordinating the stimulation of egg production and sperm storage/use in mating pairs.

2. MATERIAL AND METHODS

(a) *Fly stocks*

- (i) Wild-type stocks. Dahomey flies were as described in Chapman *et al.* (1994) and were maintained in a population cage culture. Oregon-R P2 (Allis *et al.* 1977) and Oregon R stocks were maintained in bottle culture.
- (ii) *Acp26Aa*¹ (null) and *Acp26Aa*² (truncation) flies were generated and maintained by back crossing every generation to *CyO/Df(PM101)* females as described in Herndon & Wolfner (1995). The males used in this study carried the indicated allele over *Df(2L)PM101*.

(b) *Experimental procedures*

ASG fly food medium (Gilchrist & Partridge 1997) with a few grains of live yeast added to the surface was used except where noted below. Flies were cultured at 25 ± 0.1 °C under a 12 L:12 D cycle. Dahomey flies were raised at standard densities of 50 larvae per vial. Virgin females were collected within 8 h of eclosion using ice or CO₂ anaesthesia. Flies were aged in food vials in single-sex groups of 20 for 4 days before the start of the experiment. *Acp26Aa*¹ (null) and *Acp26Aa*² (truncation) males were collected from relaxed-density, fresh bottle cultures and stored in vials for 3 days in single-sex groups of 10 before the start of the experiment. Each of 50 females was placed in a vial with two *Acp26Aa*¹ or two *Acp26Aa*² males. All flies were transferred to fresh vials after 2 days and then after 1 day, with this cycle repeated until all females were dead. The 1 day transferring was performed in order to facilitate egg counting in uncrowded vials. To ensure that males were vigorously courting females, maintain high frequencies of mating and ensure that males were fertile throughout the experiment, *Acp26Aa*¹ or *Acp26Aa*² males were replaced every 6 days until all females were dead. In each case replacement males were collected at eclosion from fresh, relaxed-density cultures and stored in single-sex groups for 3 days before introduction into the experiment. These experimental conditions of food and transfer frequency closely match those under which a survival cost of mating, if present, would be expressed (e.g. as in Chapman *et al.* 1995).

Any males that died were replaced with males of an equivalent age. Female longevity was scored every day and eggs were counted every third day from the 1 day samples. All vials were retained in order to count emerging adult progeny. The proportion of eggs that gave rise to progeny from the 1 day samples was calculated to give a measure of egg hatchability. A subset of females was similarly housed with Oregon R or Dahomey wild-type males in order to confirm that the protein produced and transferred by *Acp26Aa*² males had an effect on egg production and hatchability that was comparable with that of wild-type males. To determine the proportion of unfertilized eggs at different post-mating times, we raised Oregon R or Dahomey flies on yeast-glucose food supplemented with grains of live yeast. Eggs were collected from 100–120 females for 1 h time-periods beginning at several time points following single, within-strain matings. Eggs were fixed and stained with a DNA dye (propidium iodide (Molecular Probes, Oregon, USA) or DAPI, depending on the experiment) and a 1:400 dilution of

monoclonal anti-sperm tail antibody (Karr 1991) (antibodies kindly provided by Dr T. Karr, University of Chicago) following the procedures described in Lopez *et al.* (1994). Secondary antibodies were BODIPY FL goat anti-mouse antibodies (Molecular Probes, OR). Stained eggs were visualized on a BioRad (BioRad, Hercules, CA, USA) MRC-600 confocal microscope in order to determine the proportion that had been fertilized (i.e. contained a sperm tail).

3. RESULTS

To test the prediction that Acp26Aa transfer releases mature eggs from the ovaries before sperm are completely stored, thus resulting in lower initial egg hatchability, we compared the number of eggs laid, number of progeny produced and egg hatchability scores for females mated to *Acp26Aa*¹ (null) and *Acp26Aa*² (truncation) males. The early egg production of females housed with *Acp26Aa*¹ (null) males was significantly lower than for females housed with *Acp26Aa*² (truncation) males (figure 1a). This difference was significant in the first sample (at 3 days, Mann–Whitney *U*-test $p = 0.029$), marginally not significantly different in the second sample (at day 6, $p = 0.068$) and not significantly different in any subsequent sample. This difference in early egg production was expected from the results of previous studies (Herndon & Wolfner 1995; Heifetz *et al.* 2000) where single matings were used for testing the effect of Acp26Aa transfer. The continuous exposure design used in this study may explain why the effect of Acp26Aa on egg production was apparent for at least 3 days after initial exposure to males rather than decaying after 1 day as previously reported (Herndon & Wolfner 1995). Females were initially virgins and they may have mated on the first, second or third day of exposure to the males, thus prolonging the observed Acp26Aa effect.

Egg samples were combined over the whole experiment to give an index of lifetime egg production. Females mating with *Acp26Aa*² (truncation) males (median lifetime egg production from 1 day samples = 371.5) did not differ significantly in their lifetime egg production from females mating with *Acp26Aa*¹ (null) males (median = 333.0) (Wilcoxon χ^2 -approximation = 0.05 and $p = 0.8$). The initial difference in egg production did not translate into a significant difference in the number of progeny produced from those eggs. In fact there were no significant differences in 1 or 2 day progeny production between females mating with *Acp26Aa*¹ or *Acp26Aa*² males throughout the experiment (data not shown). The lifetime progeny totals (from combining all 1 and 2 day samples) did not differ significantly between groups (females mating with *Acp26Aa*¹ males median lifetime progeny produced = 663.0 and females mating with *Acp26Aa*² males median lifetime progeny = 680.5, $\chi^2 = 0.08$ and $p = 0.77$).

To examine the proportion of the eggs that gave rise to progeny we calculated a measure of egg hatchability as the progeny/number of eggs $\times 100$ from 1 day samples throughout the experiment. As predicted by our hypothesis the extra eggs produced as a result of Acp26Aa transfer were apparently not fertilized in mates of *Acp26Aa*² (truncation) males. Significantly more of the eggs laid by females mating with *Acp26Aa*¹ (null) males

Figure 1. (a) Median egg production per day ($\pm$ interquartile range Q1–Q3) against time in days for the first three 24 h samples of the experiment for females housed continuously from the beginning of their lives with males that did (*Acp26Aa*² or truncation males) or did not (*Acp26Aa*¹ or null males) transfer the Acp26Aa protein at mating. Females mated to *Acp26Aa*¹ (null) males laid significantly fewer eggs than mates

than *Acp26Aa*² (truncation) males hatched in the first (day 3, $p=0.049$) and second samples (day 6, $p=0.028$). There were no significant differences in subsequent samples (figure 1b). Therefore, the early (days 3 and 6) eggs laid by females mating with the *Acp26Aa*² males were significantly less fertile than those laid by mates of *Acp26Aa*¹ males. Based on control experiments (below) with Oregon R (the genetic background of the *Acp26Aa* mutants) or Dahomey flies, we believe that the extra eggs that were produced as a result of Acp26Aa transfer during this period were not fertilized or did not develop. Eggs laid by the subset of females mating with Oregon R and Dahomey wild-type males also had significantly lower egg hatchability in the first sample (day 3, $p < 0.03$) than did eggs laid by mates of *Acp26Aa*¹ males. The absence of significant differences in progeny production between females mating with *Acp26Aa*¹ or *Acp26Aa*² males makes it unlikely that the reduced egg hatchability of eggs laid by mates of *Acp26Aa*² males was due to lack of sperm transfer.

The proportion of fertilized eggs in experiments with control flies was assessed directly by visualizing the fertilizing sperm within eggs (figure 1c). We observed that a lower proportion of eggs laid soon after first matings were fertilized as compared with the fertilization rate later, after sperm had been stably stored. In 1 h collections of eggs laid after initial matings between pairs of Oregon R flies, the ratio of unfertilized to fertilized eggs was 1.5 at 3–4 h post-mating and 0.6 at 48–49 h post-mating. A similar trend was seen after initial matings by Dahomey flies. The ratio of unfertilized to fertilized eggs was again higher early after mating (0.3 at 3–4 h post-mating) than later (0.1 at 48–49 h post-mating). These results indicate that the low hatchability observed immediately after an initial mating is likely to be due to a lower egg fertilization rate at that time.

To test the prediction that the transfer of Acp26Aa does not result in a survival cost of mating, we compared the

of *Acp26Aa*² (truncation) males in the first sample ($p=0.029$) and marginally significantly fewer eggs in the second sample ($p=0.068$). There were no significant differences in egg production in the third or any of the later samples of the experiment. Each female was kept in a vial with two *Acp26Aa*¹ or *Acp26Aa*² males ($n=50$ for each group, respectively). Males were replaced from fresh cultures every 6 days until the end of the experiment. (b) Median egg hatchability (progeny/eggs $\times 100$) per day ($\pm$ interquartile range Q1–Q3) against time in days for the first three 24 h samples of the experiment for the females described in (a). The eggs laid by mates of *Acp26Aa*¹ (null) males showed significantly higher hatchability than those laid by mates of *Acp26Aa*² (truncation) males in the first ($p=0.049$) and second ($p=0.028$) samples, but not the third or any subsequent sample of the experiment. (c) The sperm tail is visible in a fertilized egg. This egg from an Oregon R female was stained with anti-sperm tail antibodies (green) and with propidium iodide (red) in order to visualize nuclei, as described in § 2b. The sperm tail assumes a coiled configuration in the fertilized egg, whereas no such structure is seen in unfertilized eggs (Karr 1991). Several loops of this structure are visible in this optical section taken on a BioRad (BioRad, Hercules, CA, USA) MRC-600 confocal microscope. The sperm nucleus (red) is also visible in this section. The edges of the egg appear green due to autofluorescence. Bar = 50 μ m.

survival of females mating with males that did (*Acp26Aa*²) and did not (*Acp26Aa*¹) transfer the Acp26Aa protein. The two groups did not differ significantly in survival (log-rank test $p=0.36$, figure 2). This suggests that Acp26Aa is not energetically costly for females to receive, perhaps because its main effect is the release of existing mature eggs and not *de novo* egg synthesis.

We confirmed that the truncated protein transferred by *Acp26Aa*² males was functionally wild type by comparing the early egg production and egg hatchability of the subset of females housed with Oregon R or Dohomey males with that of females simultaneously housed with *Acp26Aa*² males. Egg production and egg hatchability did not differ between these groups for up to 9 days after their initial matings ($p \geq 0.1$ in all tests). The *Acp26Aa*² males therefore served as an appropriate control, sharing the genetic background of the *Acp26Aa*¹ males whilst not differing significantly from wild-type males in Acp26Aa activity.

4. DISCUSSION

In this study we tested two predictions of the hypothesis that the role of Acp26Aa (ovulin) received during a mating is to trigger ovulation (Heifetz *et al.* 2000), leading to the release of previously retained mature eggs and thereby the initiation of egg production and laying. The first prediction was that Acp26Aa transfer causes the release of mature eggs from the ovaries before sperm are completely stored (or in a position to fertilize eggs), thereby resulting in a significant drop in egg hatchability following virgin matings. In later matings, the prior removal of the initial block to ovulation would have already allowed oogenesis, ovulation and sperm storage and use to be more coordinated. The second prediction was that the release of existing mature eggs is not a costly process and, therefore, the transfer of Acp26Aa *per se* should not result in a survival cost of mating for females. Our data fitted our predictions completely, as discussed below.

The transfer of Acp26Aa did cause the production of significantly more eggs shortly after the first matings to the males that transferred it, but those eggs had significantly lower hatchability than the eggs laid by mates of *Acp26Aa*¹ (null) males. Therefore, the extra eggs laid as a result of Acp26Aa transfer were apparently not fertilized. The Acp26Aa-triggered ovulation of eggs from the ovaries occurs very rapidly after mating (within 1.5 h) (Heifetz *et al.* 2000) and this is before sperm are fully stored (up to 6–9 h) (Gilbert 1981; Gromko *et al.* 1984; Tram & Wolfner 1999). This means that the initial release of mature eggs in first matings caused by the transfer of Acp26Aa occurs whilst sperm storage is incomplete. This explains our observation of inefficient early egg fertilization in mates of *Acp26Aa*² (truncation) males: the initial eggs are not as efficiently fertilized as later ovulated eggs when oogenesis, ovulation and fertilization are more coordinated. In mates of *Acp26Aa*¹ (null) males, the initial release of mature eggs from the ovary did not occur. Eggs were initially released at a slower rate, such that, by the time of their release, fertilization could occur efficiently. Therefore, their initial hatchability was significantly higher. The results from direct determination of the

Figure 2. Cumulative female survival probability against time in days for the females described in figure 1a. There was no significant difference in female survival between the two groups ($p=0.368$).

proportion of fertilized eggs at different post-mating times indicate that the rate of fertilization was lower immediately after mating. This was presumably due to incomplete sperm storage or incorrect sperm alignment within the sperm storage organs at these early times. Thus, we infer that the lower initial egg hatchability (number of progeny/number of eggs $\times 100$) in mates of males that transferred the Acp26Aa protein was due to a lower percentage of eggs that contained a sperm rather than because these eggs failed to develop for some other reason.

The mechanism we propose for the low fertilization efficiency following initial matings also explains why the effect of Acp26Aa transfer is apparent for only a few days after the initial matings. Regardless of whether an Acp26Aa-mediated initial burst of ovulation occurred, both groups of females received Acp70A, which also plays a role in stimulating egg production but most probably by regulating a different step, i.e. oogenesis (Soller *et al.* 1997, 1999) rather than ovulation (Heifetz *et al.* 2000). Oogenesis was initiated in both groups, thereby allowing egg production and fertilization to proceed in a coordinated manner with or without the initial burst of ovulation. Acp26Aa therefore appears to serve to initiate the egg production/laying process, which, once switched on, is then modulated by other molecules. Our explanation also shows that the differences in egg fertility are not explained by differences in the number or activity of sperm stored, merely the time at which sperm are fully stored relative to the rate of ovulation.

The lower initial egg hatchability we observed must occur by a different mechanism to that recently reported by Gilchrist & Partridge (2000) and Prout & Clark (2000). For example, Prout & Clark (2000) reported that egg hatchability was decreased in the 24 h following remating due to the effects of Acps on sperm capacitation or sperm use. However, in our experiments there was no difference between lifetime progeny production between mates of *Acp26Aa*¹ versus *Acp26Aa*² males, suggesting that the females had comparable sperm loads, no significant

portion of which was discounted due to incapacitation or displacement. Consistent with this, earlier experiments (Herndon & Wolfner 1995) indicated that Acp26Aa has no effect on long-term sperm storage: females mated once to 26Aa null or control males did not differ significantly in the number of progeny they produced in the subsequent 10 days (Herndon & Wolfner 1995). Moreover, the initial effects we saw in this study were following matings with virgin females. We saw no differences in egg hatchability between females mated to *Acp26Aa*¹ or *Acp26Aa*² males after their initial matings, the situation in which Prout & Clark (2000) detected an effect of Acp26Aa on egg hatchability. We conclude that the effect we report here is different in timing and mechanism from the effects reported by Gilchrist & Partridge (2000) and Prout & Clark (2000) and is due to Acp26Aa normally reducing egg hatchability following first matings because of changes in the rate of ovulation and not because of direct effects on sperm.

Our data showing that the presence or absence of Acp26Aa from their mates does not affect the longevity or reproductive success of mated females also support the hypothesis that Acp26Aa's action is to release already mature oocytes, since this would not be an energetically demanding process. We used experimental conditions under which a survival cost of mating would have been observed had it been present (e.g. Chapman *et al.* 1995). There was no evidence of a cost in females associated with having their egg production stimulated by the transfer of Acp26Aa and no evidence that Acp26Aa can explain the survival cost of mating in females. The energetic cost of Acp26Aa transfer would be expected to be less than for a protein that stimulates the *de novo* synthesis of eggs, although the Acp26Aa effect on ovulation will cause an indirect increase in oogenesis through the feedback mechanism between these two processes (Spradling 1993). The apparent short-term effects of Acp26Aa on egg production and egg laying that we observed are consistent with small or insignificant longevity costs for females resulting from Acp26Aa activity. Given that Acp26Aa transfer does not exert a negative effect on female lifespan, it is unlikely that it is selection for avoiding longevity costs that drives the rapid evolution of Acp26Aa (Aguadé *et al.* 1992; Tsaur & Wu 1997; Aguadé 1998; Tsaur *et al.* 1998). The forces that drive evolutionary change in Acp26Aa remain intriguingly elusive.

In this study there was no obvious fitness benefit conferred on males that transferred Acp26Aa or on the females that received it. Mates of *Acp26Aa*² (truncation) males had higher initial egg production, but these extra eggs were not fertilized. Assuming that these eggs were fertilization competent, males did not capitalize on this potential increase in reproductive success. However, conditions in the field and even under normal laboratory culture in crowded bottles may be very different from those employed in this study. In the field for example, mates, food and oviposition sites are likely to be both scarce and ephemeral (e.g. Boulétreau-Merle 1978). Females capable of high rates of egg production when oviposition sites become available would be at an advantage. In the absence of mating, egg production is a wasted effort, so a signal from males that caused the removal of a block to oviposition thus allowing egg

production to proceed quickly following the receipt of sperm would be favoured. The removal of a cohort of mature eggs will also speed the development of the next eggs in the queue. It could prove costly in terms of time wasted if females relied on males to provide a signal for initiating oogenesis (the initial production of eggs); instead females appear to be primed for immediate and continual egg production. In this study, we provided females with food and males on a regular, predictable basis. Therefore, unlike in the situation just described, females were not tested for their ability to capitalize when mates and food were scarce; this may be the reason why we did not observe benefits of Acp26Aa transfer.

The trigger provided by the transfer of Acp26Aa in field conditions may also interact in a selectively favourable way with the egg retention phenotype exhibited by virgin females (Boulétreau-Merle *et al.* 1989). This genetic capacity is reported to exhibit seasonal and geographical clinal variation (Boulétreau-Merle *et al.* 1992). The trait is interpreted as a physiological capacity for delaying egg deposition when no partner is available (Boulétreau-Merle *et al.* 1992). Long egg retention phenotypes are associated with temperate habitats in which resources are unpredictable. Thus, the capacity of females for delaying egg laying in the absence of a mate or oviposition site is advantageous. However, the flip side is that females must also have the capacity for initiating egg laying quickly once mating has occurred. This is achieved by the transfer of Acp26Aa, which then has a crucially important role in triggering the initiation of egg production and egg laying. In this study, the females used were from a single wild-type stock and, thus, any interaction between Acp26Aa and egg retention would not have been seen.

If eggs retained for a long time in the virgin female become 'stale', that is less capable of being fertilized or of supporting development, it would be to the advantage of males and females to facilitate the quick removal of these eggs following mating by using a molecule such as Acp26Aa. This would avoid wasting sperm before fertilization-competent eggs become available. However, our data argue that this is not the most likely function for Acp26Aa. That the hatchability of eggs from mates of *Acp26Aa*¹ males was higher than that of mates of *Acp26Aa*² males suggests that the eggs that were not cleared out in the former females were capable of supporting development; our data cannot address whether 'stale' eggs develop at the same rate as eggs that had not been 'held' in the ovaries prior to fertilization. It would be interesting to investigate the fertilization and developmental rates of eggs retained in the ovaries of unmated laboratory and field females further.

Acp26Aa is the most rapidly evolving protein in the *D. melanogaster* genome yet reported (Ting *et al.* 1998; W. J. Swanson, personal communication). The *Acp26Aa* locus shows evidence of rapid evolutionary change, both within and between species (Aguadé *et al.* 1992; Tsaur & Wu 1997; Aguadé 1998; Tsaur *et al.* 1998). The functional significance of *Acp26Aa* allelic variants is not yet known, but it is tempting to consider the possibility that different alleles produce quantitatively different effects on ovulation. This could be driven by sexual selection contests between males for fertilizations or, alternatively, by the different

genetic interests of males and females per mating (Chapman & Partridge 1996).

A very important finding from this study is that timing is everything. We showed that the receipt of Acp26Aa during initial matings released mature eggs from the ovaries before sperm were completely stored, resulting in lower initial egg hatchability. We suggest that Acp26Aa functions to remove a block to oogenesis by clearing out existing mature eggs and, thus, indirectly allowing oogenesis to be initiated immediately after mating. The magnitude of the Acp26Aa-mediated effect on early egg production was significant but subtle and did not result in a significant increase in female lifetime reproductive success. This suggests that the initial burst of ovulation in response to Acp26Aa transfer was followed by slightly lower (but statistically insignificant) egg laying in several subsequent samples. The receipt of Acp26Aa did not result in any obvious fitness costs for females, consistent with the idea that this protein serves to clear out eggs and does not act as a direct stimulus for initiating the potentially costly process of oogenesis. The potential for interactions between Acps that affect different specific aspects of egg production and the responses of females to those Acps certainly merits further investigation.

We thank the Human Frontiers Science Project for early funding that led to the conception of this study and The Royal Society (university research fellowship to T.C.), Biotechnology and Biological Sciences Research Council, Natural Environment Research Council (professorship to L.P.), National Science Foundation (grants IBN97-23356 and IBN99-04824 to M.F.W.) and National Institutes of Health (training grant T32-GM07617 for support of L.A.H. during part of these studies) for financial support of this work. We are grateful to Dr T. Karr for anti-sperm tail antibodies and to participants of the Sixth International Seminal Peptides Meeting at Cornell University for very helpful discussions.

REFERENCES

- Aguadé, M. 1998 Different forces drive the evolution of the *Acp26Aa* and *Acp26Ab* accessory gland genes in the *Drosophila melanogaster* species complex. *Genetics* **150**, 1079–1089.
- Aguadé, M. 2000 Positive selection drives the evolution of the *Acp29AB* accessory gland protein in *Drosophila*. *Genetics* **152**, 543–551.
- Aguadé, M., Miyashita, N. & Langley, C. H. 1992 Polymorphism and divergence in the *Mst26A* male accessory gland gene region in *Drosophila*. *Genetics* **132**, 755–770.
- Aigaki, T., Fleischmann, I., Chen, P. S. & Kubli, E. 1991 Ectopic expression of sex peptide alters reproductive behaviour of female *D. melanogaster*. *Neuron* **7**, 1–20.
- Allis, C. D., Waring, G. L. & Mahowald, A. P. 1977 Mass isolation of pole cells from *Drosophila melanogaster*. *Dev Biol.* **56**, 372–381.
- Boulétreau-Merle, J. 1978 Ovarian activity and reproductive potential in a natural population of *Drosophila melanogaster*. *Oecologia (Berlin)* **35**, 319–342.
- Boulétreau-Merle, J., Terrier, O. & Fouillet, P. 1989 Chromosomal analysis of initial retention capacity in virgin *Drosophila melanogaster* females. *Heredity* **62**, 145–151.
- Boulétreau-Merle, J., Fouillet, P. & Terrier, O. 1992 Clinal and seasonal variations in initial retention capacity of virgin *Drosophila melanogaster* females as a strategy for fitness. *Evol. Ecol.* **6**, 223–242.
- Chapman, T. & Partridge, L. 1996 Sexual conflict as fuel for evolution. *Nature* **381**, 189–190.
- Chapman, T., Trevitt, S. & Partridge, L. 1994 Remating and male-derived nutrients in *Drosophila melanogaster*. *J. Evol. Biol.* **7**, 51–69.
- Chapman, T., Liddle, L. F., Kalb, J. M., Wolfner, M. F. & Partridge, L. 1995 Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature* **373**, 241–244.
- Chen, P. S., Stumm-Zollinger, E., Aigaki, T., Balmer, J., Bienz, M. & Böhlen, P. 1988 A male accessory gland peptide that regulates reproductive behaviour of female *D. melanogaster*. *Cell* **54**, 291–298.
- Civetta, A. 1999 Direct visualization of sperm competition and sperm storage in *Drosophila*. *Curr. Biol.* **9**, 841–844.
- DiBenedetto, A. J., Lakich, D. M., Kruger, W. D., Belote, J. M., Baker, B. S. & Wolfner, M. F. 1987 Sequences expressed sex-specifically in *Drosophila melanogaster* adults. *Dev Biol.* **119**, 242–251.
- Eberhard, W. G. 1996 *Female control: sexual selection by cryptic female choice*. Princeton University Press.
- Gilbert, D. G. 1981 Ejaculate esterase 6 and initial sperm use by female *Drosophila melanogaster*. *J. Insect Physiol.* **27**, 641–650.
- Gilchrist, A. S. & Partridge, L. 1997 Heritability of pre-adult viability differences can explain apparent heritability of sperm displacement ability in *Drosophila melanogaster*. *Proc. R. Soc. Lond. B* **264**, 1271–1275.
- Gilchrist, A. S. & Partridge, L. 2000 Why it is difficult to model sperm displacement in *Drosophila melanogaster*: the relation between sperm transfer and copulation duration. *Evolution* **54**, 534–542.
- Gromko, M. H., Gilbert, D. G. & Richmond, R. C. 1984 Sperm transfer and use in the multiple mating system in *Drosophila*. In *Sperm competition and the evolution of animal mating systems* (ed. R. L. Smith), pp. 371–426. New York: Academic Press.
- Harshman, L. G. & Clark, A. G. 1998 Inference of sperm competition from broods of field-caught *Drosophila*. *Evolution* **52**, 1334–1341.
- Heifetz, Y., Lung, O., Frongillo, E. A. & Wolfner, M. F. 2000 The *Drosophila* seminal fluid protein Acp26Aa stimulates release of oocytes by the ovary. *Curr. Biol.* **10**, 99–102.
- Herndon, L. A. & Wolfner, M. F. 1995 A *Drosophila* seminal fluid protein, Acp26Aa, stimulates egg-laying in females for 1 day after mating. *Proc. Natl Acad. Sci. USA* **92**, 10 114–10 118.
- Hihara, F. 1981 Effects of the male accessory gland secretion on oviposition and remating in females of *Drosophila melanogaster*. *Zool. Mag.* **90**, 303–316.
- Imhof, M., Harr, B., Brem, G. & Schlötterer, C. 1998 Multiple mating in wild *Drosophila melanogaster* revisited by microsatellite analysis. *Mol. Ecol.* **7**, 915–917.
- Kalb, J., DiBenedetto, A. J. & Wolfner, M. F. 1993 Probing the function of *Drosophila melanogaster* accessory glands by directed cell ablation. *Proc. Natl Acad. Sci. USA* **90**, 8093–8097.
- Karr, T. L. 1991 Intracellular sperm/egg interactions in *Drosophila*: a three-dimensional structural analysis of a paternal product in the developing egg. *Mech. Dev.* **34**, 101–111.
- Lopez, J., Song, K., Hirshfeld, A., Lin, H. & Wolfner, M. F. 1994 The *Drosophila* fs(1)Ya protein, which is needed for the first mitotic division, is in the nuclear lamina and in the envelopes of cleavage nuclei, pronuclei and nonmitotic nuclei. *Dev Biol.* **163**, 202–211.
- Lung, O., Tram, U., Finnerty, L. M., Eipper-Mains, M., Kalb, J. M. & Wolfner, M. F. 2001 The *Drosophila melanogaster* seminal fluid protein *Acp62F* is a protease inhibitor that is toxic upon ectopic expression. *Genetics*, submitted.
- Manning, A. 1962 A sperm factor affecting the receptivity of *Drosophila melanogaster* females. *Nature* **194**, 252–253.

- Manning, A. 1967 The control of sexual receptivity in female *Drosophila*. *Anim. Behav.* **15**, 239–250.
- Monsma, S. A. & Wolfner, M. F. 1988 Structure and expression of a *Drosophila* male accessory gland gene whose product resembles a peptide prehormone precursor. *Genes Dev.* **2**, 1063–1073.
- Moshitzky, P., Fleischmann, I., Chaimov, N., Saudan, P., Klauser, S., Kubli, E. & Applebaum, S. W. 1996 Sex-peptide activates juvenile hormone biosynthesis in the *Drosophila melanogaster corpus allatum*. *Arch. Insect Biochem. Physiol.* **32**, 363–374.
- Neubauer, D. M. & Wolfner, M. F. 1999 Mated *Drosophila* females require a seminal fluid protein, Acp36DE, to store sperm efficiently. *Genetics* **153**, 845–857.
- Parker, G. A. 1970 Sperm competition and its evolutionary consequences in the insects. *Biol. Rev.* **45**, 525–567.
- Partridge, L., Green, L. & Fowler, K. 1987 Effects of egg production and of exposure to males on female survival in *Drosophila melanogaster*. *J. Insect Physiol.* **33**, 746–749.
- Price, C. S. C., Dyer, K. A. & Coyne, J. A. 1999 Sperm competition between *Drosophila* males involves both displacement and incapacitation. *Nature* **400**, 449–452.
- Prout, T. & Clark, A. G. 2000 Seminal fluid causes temporary reduced egg hatch in previously mated females. *Proc. R. Soc. Lond. B* **267**, 201–203.
- Schäfer, U. 1986 Genes for male-specific transcripts in *Drosophila melanogaster*. *Mol. Gen. Genet.* **202**, 219–225.
- Sgrò, C. M. & Partridge, L. 1999 A delayed wave of death from reproduction in *Drosophila*. *Science* **286**, 2521–2524.
- Simmons, L. & Siva-Jothy, M. 1998 Sperm competition in the insects: mechanisms and the potential for selection. In *Sperm competition and sexual selection* (ed. T. R. Birkhead & A. P. Møller), pp. 341–434. Cambridge University Press.
- Soller, M., Bownes, M. & Kubli, E. 1997 Mating and sex peptide stimulate the accumulation of yolk in oocytes of *Drosophila melanogaster*. *Eur. J. Biochem.* **243**, 732–738.
- Soller, M., Bownes, M. & Kubli, E. 1999 Control of oocyte maturation in sexually mature *Drosophila* females. *Dev. Biol.* **208**, 337–351.
- Spradling, A. C. 1993 Developmental genetics of oogenesis. In *The development of Drosophila melanogaster*, vol. I. (ed. M. Bate & A. Martinez-Arias), pp. 1–70. Cold Spring Harbor, NY: Cold Spring Harbor Press.
- Stumm-Zollinger, E. & Chen, P. S. 1985 Protein metabolism of male accessory glands. I. Characterisation of secretory proteins. *Insect Biochem.* **15**, 375–383.
- Swanson, W. J., Clark, A. G., Waldrip-Dail, H. M., Wolfner, M. F. & Aquadro, C. F. 2001 Evolutionary EST analysis identifies adaptive evolution in *Drosophila* male reproductive proteins. PNAS (In the press.)
- Ting, C. T., Tsaur, S. C., Wu, M. L. & Wu, C.-I. 1998 A rapidly evolving homeobox at the site of a hybrid sterility gene. *Science* **282**, 1501–1504.
- Tram, U. & Wolfner, M. F. 1999 Male seminal fluid proteins are essential for sperm storage in *Drosophila melanogaster*. *Genetics* **153**, 837–844.
- Tsaur, S. C. & Wu, C.-I. 1997 Positive selection and the molecular evolution of a gene of male reproduction, *Acp26Aa* of *Drosophila*. *Mol. Biol. Evol.* **14**, 544–549.
- Tsaur, S. C., Ting, C. T. & Wu, C.-I. 1998 Positive selection driving the evolution of a gene of male reproduction, *Acp26Aa*, of *Drosophila*: II. Divergence versus polymorphism. *Mol. Biol. Evol.* **15**, 1040–1046.
- Wolfner, M. F. 1997 Tokens of love: functions and regulation of *Drosophila* male accessory gland products. *Insect Biochem. Mol. Biol.* **27**, 179–192.
- Wolfner, M. F., Harada, H. A., Bertram, M. J., Stelick, T., Kraus, K. W., Kalb, J. M., Lung, Y. O., Neubauer, D. M., Park, M. & Tram, U. 1997 New genes for male accessory gland proteins in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* **27**, 825–834.
- Xue, L. & Noll, M. 2000 *Drosophila* female sexual behavior induced by sterile males showing copulation complementation. *Proc. Natl Acad. Sci. USA* **97**, 3272–3275.

As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.