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The role of male accessory gland protein Acp36DE in sperm competition in *Drosophila melanogaster*

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A crucial factor determining sperm fertilization success in multiply mated *Drosophila melanogaster* females is the efficiency with which sperm are stored. This process is modulated by the accessory gland protein Acp36DE. In this study, we show that the effect of Acp36DE on sperm storage itself alters the outcome of sperm competition. As second-mating males, *Acp36DE*^{1} (null) males had significantly lower P_2-values than *Acp36DE*^{2} (truncation) or *Acp36DE*$^+$ (control) males, as might be expected as the null males' sperm are poorly stored. We used spermless males, which are null for Acp36DE, to show that, in the absence of sperm co-transfer, Acp36DE itself could not displace first-male sperm. The results therefore suggest that males null for Acp36DE suffer in sperm displacement because fewer sperm are stored or retained, not because Acp36DE itself displaces sperm. *Acp36DE*^{1} (null) males also gained significantly fewer fertilizations than controls when they were the first males to mate. Using spermless males, we also showed that significantly more second-male offspring were produced following the transfer of Acp36DE by spermless first-mating males. This implies that the transfer of Acp36DE itself by the first male facilitated the storage or use of the second male's sperm and that co-transfer with sperm is not necessary for Acp36DE effects on second-male sperm storage. Acp36DE may persist in the reproductive tract and aid the storage of any sperm including those of later-mating males or prime the female for future efficient sperm storage. Our results indicate that mutations in genes that affect sperm storage can drastically affect the outcome of sperm competition.

Keywords: sperm competition; sperm storage; Acp36DE; *Drosophila melanogaster*

1. INTRODUCTION

Sperm competition is widespread in insects (Boorman & Parker 1976; Gwynne 1984; Ridley 1988; Simmons & Siva-Jothy 1998) as a result of the presence of ejaculates from more than one male in the female reproductive tract (Parker 1970). Studies using genetic markers have produced evidence for concurrent multiple paternity of offspring in *Drosophila melanogaster* in nature (e.g. Milkman & Zeitler 1974; Ochando *et al*. 1996; Harshman & Clark 1998; Imhof *et al*. 1998). Success in sperm competition is therefore an important component of male reproductive success in this species. Both the defence of sperm in storage against displacement or inactivation by ejaculates from later matings (defence) and the ability of an ejaculate to displace or inactivate sperm already in storage (offence) are important determinants of success in sperm competition (Service & Fales 1993; Harshman & Prout 1994). Defence can be measured by the proportion of first-male sperm used to fertilize progeny following a second mating (P_1) and displacement by the proportion of second-male sperm used (P_2) (Boorman & Parker 1976). Ejaculates are expected to be in strong competition, but facilitatory effects of one ejaculate upon another are possible, although this idea has not been previously tested.

The number of sperm transferred, remating interval, current female fecundity and the proteins synthesized by the male accessory glands (Acps) can all affect the success of sperm in competition (reviewed by Simmons & Siva-Jothy 1998). However, the exact mechanisms by which ejaculates interact and compete in *D. melanogaster* are still poorly understood (Gilchrist & Partridge 2000). There have been two main areas of study: first, the population and quantitative genetics of sperm defence and displacement (e.g. Clark *et al*. 1995; Prout & Clark 1996; Hughes 1997) and, second, the dissection of sperm storage, defence and displacement mechanisms using mutants or transgenic flies and 'interrupted' matings to prevent sperm or accessory gland product transfer (e.g. Milkman & Zeitler 1974; Scott & Richmond 1990; Kalb *et al*. 1993; Harshman & Prout 1994; Ochando *et al*. 1996; Civetta 1999; Neubaum & Wolfner 1999; Price *et al*. 1999; Tram & Wolfner 1999; Gilchrist & Partridge 2000). Female genotype (Birkhead 1998; Clark & Begun 1998) and male–female interactions (Price 1997; Arthur *et al*. 1998; Clark *et al*. 1999) can also influence which sperm succeed.

Dissection of the mechanisms underlying sperm competition has shown that displacement or inactivation of sperm can be achieved by both sperm and seminal fluid (e.g. Scott & Richmond 1990; Harshman & Prout 1994; Price *et al*. 1999; Gilchrist & Partridge 2000; Prout & Clark 2000). Displacement by seminal fluid (comprising the products of the cells in the accessory glands, ejaculatory duct and bulb) has been inferred from the results of most studies which used genetically spermless males (Scott & Richmond 1990; Price *et al*. 1999; Gilchrist & Partridge

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2000); although one study (Gromko *et al.* 1984) did not detect displacement in the absence of transferred sperm. The seminal fluid components which mediated this effect were shown to be accessory gland products by Harshman & Prout (1994). Effective sperm storage is also dependent on the transfer of accessory gland products (Tram & Wolfner 1999).

Displacement, presumably by seminal fluid, within 6 h after spermless rematings with *XO* males has been reported (Scott & Richmond 1990). Interrupting rematings before sperm transfer or rematings with spermless males provides evidence for displacement or inactivation after ten to 12 days (Harshman & Prout 1994) or two days (Gilchrist & Partridge 2000) or three days (Prout & Clark 2000) and after seven but not two days (Price *et al.* 1999). Price *et al.* (1999) concluded that success in competition can be achieved by displacement, due to the presence of sperm, and inactivation due to the effects of seminal fluid seven days after remating. The reasons for the discrepant results at two to three days (Price *et al.* 1999; Gilchrist & Partridge 2000; Prout & Clark 2000) are not clear. They could be attributable to differences in the statistical power in the three studies or to variation in the time allotted for rematings (which may have resulted in differences in the proportion of second-mated females in which displacement could potentially occur) or to the different stocks used. In terms of the latter, the green fluorescent protein (GFP)-labelled sperm which allowed Price *et al.* (1999) to make direct observations of sperm in storage are impaired in their overall efficiency of transfer/storage, which could have contributed to the differences observed between the studies. Current evidence suggests that, although seminal fluid can displace or inactivate sperm, the larger displacement seen in normal matings also requires the transfer of sperm (Price *et al.* 1999; Gilchrist & Partridge 2000).

It has recently been shown using transgenic males which lack Acps that accessory gland proteins are necessary for sperm storage, which is likely to be a key component of sperm competition (Kalb *et al.* 1993; Tram & Wolfner 1999). It is unknown how many Acps are involved in these processes, but Acp36DE is of particular interest in terms of a potential effect on sperm competition because of its essential role in sperm storage (Neubaum & Wolfner 1999). In recently mated females, the Acp36DE protein accumulates at a site in the lower oviduct, just above the sperm storage organs (the paired spermathecae and the seminal receptacle) and associates tightly with sperm and the sperm mass (Bertram *et al.* 1996; Neubaum & Wolfner 1999). During the time of sperm storage, Acp36DE enters the spermathecae and the seminal receptacle (Neubaum & Wolfner 1999). Although males specifically lacking Acp36DE make and transfer normal amounts of motile sperm, those sperm fail to accumulate properly in the sperm storage organs (only 15% of the wild-type number of sperm are stored soon after mating). This failure could be because fewer sperm enter into storage or because fewer are retained in storage (Neubaum & Wolfner 1999). Males null for Acp36DE but transferring sperm would not be predicted to do well in defence or displacement because their sperm are poorly stored. However, this has not been previously investigated. In addition, the interaction between Acp36DE and sperm co-transfer, i.e. its effect on sperm competition in the absence of co-transferred sperm, is not known.

Population studies have also suggested that Acp36DE plays a role in the process of sperm competition. In tests of 152 lines of flies originally isolated from natural populations, Clark *et al.* (1995) identified statistically significant associations between sperm defence ability and allelic variation at four loci including *Acp36DE*. No such association was observed between variation in *Acp36DE* and the ability of males to displace sperm. However, the possibility that the association detected by Clark *et al.* (1995) is due not to the *Acp36DE* locus but to an unidentified locus in linkage disequilibrium with it could not be discounted.

The aim of this study was to investigate the involvement of Acp36DE in sperm competition. We tested how the ejaculates of different males interact with one another, both in the presence and absence of sperm, in determining the extent of sperm displacement and defence. To investigate sperm displacement, *cn bw* females were first mated to *cn bw* males and then, after 48 h, to males which transferred normal seminal fluid ($Acp36DE^{+}$ (control) males), seminal fluid lacking the Acp36DE protein ($Acp36DE^{1}$ (null) males) or seminal fluid in which the Acp36DE protein was truncated but still functional in sperm storage ($Acp36DE^{2}$ (truncation) males). This allowed comparisons of the effects of different second males on first-male progeny production. We then tested whether Acp36DE itself could displace sperm of the first-mating males using irradiated and, therefore, spermless males which produced or lacked Acp36DE as second mates. To investigate sperm defence, *cn bw* females were first mated to $Acp36DE^{+}$ $Acp36DE^{1}$ or $Acp36DE^{2}$ males and then remated after one or two days to *cn bw* males. This permitted the effect of the genotype of the first male on first- and second-male progeny production after remating to be determined. To test the influence of Acp36DE itself on progeny production by second-mating males, we again used irradiated males. Wild-type females were first mated to spermless $Acp36DE^{1}$ or $Acp36DE^{+}$ males. After 24 or 48 h, females were remated with wild-type males, allowing comparisons of the effects of Acp36DE transfer from first males on second-male progeny production.

Our results showed that $Acp36DE^{1}$ (null) males did poorly in fertilizations as second-mating males. They achieved significantly lower P_2-values than $Acp36DE^{+}$ (control) or $Acp36DE^{2}$ (truncation) males, presumably because fewer of their sperm were stored. There was no actual displacement of first-male sperm by Acp36DE transfer in the absence of sperm. $Acp36DE^{1}$ males also did poorly as first-mating males, again because fewer of their sperm were initially stored. In the absence of sperm transfer by first-mating males, Acp36DE facilitated the storage or use of the second male's sperm.

2. MATERIAL AND METHODS

The fly stocks and mutants used are described below.

(i) Dahomey wild-type. These flies were from the Dahomey wild-type stock collected in 1970 in Dahomey (now Benin) and maintained in population cage culture (described in Chapman *et al.* 1994).

(ii) A *cn bw* stock, which was described in Clark *et al.* (1995), was kindly provided by Dr Andrew Clark. *cn bw* flies allowed us to determine the paternity of first- and second-mating male progeny in experiments where both males transferred sperm. Of the offspring from the *cn bw* mothers, those that were homozygous for *cn bw* had white eyes, while heterozygotes had wild-type eyes.

(iii) *Acp36DE*^{1} (Acp36DE null), *Acp36DE*^{2} (Acp36DE truncation mutant) and *Acp36DE*$^+$ (control) were as described in Neubaum & Wolfner (1999). The mutant chromosomes were derived from lines originally isogenic for chromosome 2 which had been treated with ethyl methanesulphonate. They were identified as mutant based on the loss or truncation of the Acp36DE protein in hemizygous males. Acp36DE null (*Acp36DE*^{1}), truncation (*Acp36DE*^{2}) or control (*Acp36DE*$^+$) chromosomes were maintained by backcrossing every generation to *CyO/Df(2L)H*$_2$O females (a deficiency which deletes the *Acp36DE* locus). The flies used in our experiments carried the *Acp36DE* allele indicated over *Df(2L)H*$_2$O. The truncated Acp36DE protein produced by *Acp36DE*^{2} males contains all the sequences required for normal activity. It is also present at sufficient levels and is indistinguishable in activity from the Acp36DE protein produced by the wild-type allele in all tests to date (Neubaum & Wolfner 1999; this study).

(iv) *XO* males. An attached X stock with a Dahomey genetic background was made by chromosome substitution (described in Chapman 1992). *XO* males were obtained by crossing Dahomey virgin females to attached *XY* males. Mating *XO* males transfer no sperm (Keifer 1966; Hardy *et al.* 1981; Gilchrist & Partridge 1995), but sperm are not necessary for Acp transfer (U. Tram and M. F. Wolfner, unpublished data).

(v) *DTA* males were from a transgenic stock (*DTA-E*) in which the coding sequence for the diptheria toxin subunit A is under the control of an accessory gland main cell-specific promoter (Kalb *et al.* 1993). These males produce this intracellular toxin in their accessory gland main cells, preventing the production of any detectable Acps by these cells. *DTA-E* males also do not produce sperm, but their secondary cell, ejaculatory duct and ejaculatory bulb molecule synthesis and transfer are unaffected (Kalb *et al.* 1993). As described by Kalb *et al.* (1993), *DTA-E* males are maintained by backcrossing to *ry*^{506} which, for the present study, had been crossed into a Dahomey wild-type background to provide *DTA* males with a genetically similar background to the *XO* males described above.

Experiments involving non-irradiated males were done on standard yeast–glucose fly medium seeded with live yeast. The flies in these experiments were maintained at 24 ± 0.5 °C under a 12 L:12 D cycle. In the other experiments, ASG fly food medium (Gilchrist & Partridge 1997) with a few grains of live yeast added to the surface was used. In the experiments with irradiated males, the flies were kept at 25 ± 0.1 °C under a 12 L:12 D cycle.

(a) Acp36DE *and displacement* (Acp36DE^1 (*null*), Acp36DE^2 (*truncation*) *and* Acp36DE$^+$ (*control*) *males as second males*)

(i) *Acp36DE*^{1}*, Acp36DE*^{2} *or Acp36DE*$^+$ *males, with sperm*

To investigate the role of the *Acp36DE* protein in displacing sperm already in storage, *cn bw* females were first mated to *cn bw* males and then remated with *Acp36DE*^{1} (null), *Acp36DE*^{2} (truncation) or *Acp36DE*$^+$ (control) males using the method of Clark *et al.* (1995). *cn bw* females were collected shortly after eclosion (0–2 h) on ice and aged for three to five days on standard fly food. Approximately six *cn bw* females and seven to nine *cn bw* males were placed together in fresh food vials and allowed to mate. Mating pairs were removed by aspiration and transferred to a fresh food vial (vial 1). The males were removed within 30 min after copulation finished. Individual females remained in these vials for two days. Two each of the *Acp36DE*^{1}, *Acp36DE*^{2} or *Acp36DE*$^+$ males were then introduced into each vial. Females and males were left together overnight (*ca.* 18 h) after which time the males were removed and females aspirated into a fresh vial (vial 2). Each vacated vial 1 thus predominantly contained the offspring of male 1, together with the first few of male 2's offspring produced after the second mating in the overnight period before transfer to vial 2. On the sixth day after the first mating, females were tapped into fresh food vials (vial 3) and maintained in this vial for one week. Vial 2 therefore contained the offspring of the first and second males produced over the six-day period after the second mating. The number and eye colour of all adult progeny in vials 1–3 were scored when all progeny had emerged (usually *ca.* 12 days after the last eggs were laid).

(ii) *Acp36DE*^{1} *or Acp36DE*$^+$ *males, lacking sperm*

To investigate whether any effect of the Acp36DE protein on displacement is dependent on its co-transfer with sperm, we used spermless *Acp36DE*^{1} (null) and *Acp36DE*$^+$ (control) males as second mates. Dahomey flies were raised at standard larval densities (100 larvae per vial). *Acp36DE*^{1} and *Acp36DE*$^+$ males were collected from relaxed density cultures at eclosion and sterilized at five to ten days old with 10 kRad of X-irradiation, as described in Gilchrist & Partridge (1995). Following irradiation, the males were placed in groups of five together with ten virgin females for three days. The males mated multiple times during this period, which exhausted their residual, sterile sperm. The males were then separated from the females, placed together in groups of 30 and allowed 48 h to recover, allowing their accessory glands to fully resynthesize Acps (DiBenedetto *et al.* 1990; Herndon *et al.* 1997). Subsets of these males were tested for sterility by mating them individually with virgin females; no progeny resulted from these matings. Males subjected to such irradiation have previously been shown to be indistinguishable from genetically spermless males in the degree to which they reduce virgin female receptivity (Gilchrist & Partridge 1995) and in their sperm displacement characteristics (Gilchrist & Partridge 2000).

Using aspiration throughout, three- to five-day-old, virgin, wild-type females were placed one per vial with one wild-type male each until mating occurred; the males were removed within 30 min of the end of copulation. Three days later, the females were remated with either irradiated *Acp36DE*^{1} (null) or *Acp36DE*$^+$ (control) males. Unmated females were remated the following day (four days after the initial matings) or the next day (five days after the initial matings). Females were transferred into new food vials every two days and vacated vials were retained to count the emerging progeny within two to five days of their eclosion.

(b) Acp36DE *and defence* (Acp36DE^1 (*null*), Acp36DE^2 (*truncation*) *and* Acp36DE$^+$ (*control*) *males as first males*)

(i) *Acp36DE*^{1}*, Acp36DE*^{2} *and Acp36DE*$^+$ *males, with sperm*

To investigate the effect of Acp36DE in defence against displacement, *cn bw* females were first mated to *Acp36DE*^{1} (null), *Acp36DE*^{2} (truncation) or *Acp36DE*$^+$ (control) males and

remated 24 or 48 h later to *cn bw* males using a method similar to that described in § 2(a)(i). Three replicate experiments were performed and the number and eye colour of emerging progeny in vials 1–3 were recorded for each female. Rematings were performed 48, 48 and 24 h after the first matings for replicates 1, 2 and 3, respectively.

(ii) *Acp36DE^1 and Acp36DE$^+$ males, lacking sperm*

To investigate whether first-male Acp36DE transfer affects sperm storage or use by second-mating males, we used irradiated, spermless *Acp36DE*^{1} and *Acp36DE*$^+$ males as first mates. Three- to five-day-old, Dahomey, wild-type, virgin females were first mated to irradiated *Acp36DE*^{1} or *Acp36DE*$^+$males. The following day the females were remated with wild-type males. Females which did not remate were remated 24 h later. The females were then transferred into new food vials every two days and the vacated vials retained to count the emerging progeny.

To provide an additional test of the effects of first-male Acp36DE on second-male progeny production in the absence of first-male sperm co-transfer, we tested independently derived males which lacked all main cell Acps, including Acp36DE. We compared the number of progeny produced by females first mated with *XO* (full Acps but no sperm) or *DTA* (no main cell Acps or sperm) males and then to wild-type males. Since *XO* and *DTA* males both fail to transfer sperm at mating, irradiation treatment was omitted. Females were first mated to *XO* or *DTA* males and observed until mating took place. Unlike the *Acp36DE*^{1} (null) and *Acp36DE*$^+$ (control) males in the previous experiment, *XO* and *DTA* males also differ in whether they transfer Acps which stimulate egg production, e.g. Acp70A (the sex peptide) (Chen *et al*. 1988) and Acp26Aa (ovulin) (Herndon & Wolfner 1995; Heifetz *et al*. 2000). This difference, if not controlled, could potentially confound the results because egg production and sperm usage are correlated (Trevitt *et al*. 1988). To control for this, we injected all females with physiological amounts of synthetic sex peptide (Acp70A) (Chen *et al*. 1988) starting 1 h after the *XO* or *DTA* first matings. Each female was anaesthetized on ice and injected with 5 pmol sex peptide (donated generously by E. Kubli) in 50 nl phosphate-buffered saline (Maniatis *et al*. 1982). It was not possible to co-inject Acp26Aa which has an independent stimulatory effect on ovulation (Herndon & Wolfner 1995; Heifetz *et al*. 2000).

The next morning, females were placed one per vial with two Dahomey males each until mating took place. The majority of females did not remate on this day (47 out of 62 had not remated after *XO* first matings and 36 out of 71 after *DTA* first matings); significantly more females did not remate following first matings to *XO* males, ($\chi^2_{\text{Yates}} = 7.85$ and $p < 0.005$). We attributed this difference to the known receptivity-reducing effect of Acp70A (Chen *et al*. 1988). Females mated to *XO* males received Acp70A from mating and the injections, while females mated to *DTA* males received Acp70A from the injections only. On the following day, 84% of females first mated to *XO* males and 90% of females first mated to *DTA* males remained remated. The females were then transferred into new food vials every two days and the number of progeny produced by each female was recorded.

(c) *Statistical analysis*

Shapiro–Wilk tests were used to test the data for normality (Zar 1996). The data were highly non-normal in many cases and non-parametric tests (Kruskal–Wallis and Wilcoxon tests) were used for data analysis (Zar 1996). Vials in which zero progeny were produced, remating did not occur or the female died were excluded from analysis in experiments using *Acp36DE*^{1}, *Acp36DE*^{2} and *Acp36DE*$^+$ males with sperm. In the other experiments, counts from vials in which females died or failed to mate or remate were excluded. All analysis was performed using JMP statistical software for a Macintosh computer (SAS Institute Inc., Cary, NC, USA).

3. RESULTS

(a) Acp36DE *and displacement* (Acp36DE^1 (*null*), Acp36DE^2 (*truncation*) *and* Acp36DE$^+$ (*control*) *males as second males*)

(i) *Acp36DE^1, Acp36DE^2 or Acp36DE$^+$ males, with sperm*

We analysed the number of progeny fathered by first-mating *cn bw* males and second-mating *Acp36DE*^{1}, *Acp36DE*^{2} and *Acp36DE*$^+$ males. Vial 1 contained nearly all progeny of the first-mating male and the total number of progeny did not differ between groups (Kruskal–Wallis test $p = 0.23$). The total number of progeny in vials 2 and 3 combined (i.e. nearly all of the progeny produced after the second mating) differed significantly between groups due to a significantly higher progeny output by second mates of *Acp36DE*^{2} (truncation) and *Acp36DE*$^+$ (control) relative to *Acp36DE*^{1} (null) males ($p = 0.0018$). Since the differences in total progeny therefore appeared to be male determined, to avoid potentially confounding the P_2-values with this effect we analysed only the progeny produced in vial 2 (those produced in the six days from shortly after the second matings); these did not differ in total progeny counts between groups ($p = 0.11$). However, there were significant differences between the number of first- ($p = 0.0039$) and second-male ($p = 0.0004$) progeny produced in vial 2 by females first mated to *Acp36DE*^{1} relative to *Acp36DE*^{2} or *Acp36DE*$^+$ males (figure 1*a*). This is shown more clearly as a significantly lower P_2-value ($p < 0.0002$) for *Acp36DE*^{1} compared with *Acp36DE*^{2} and *Acp36DE*$^+$ males, which did not differ from one another (figure 1*b*). *Acp36DE*^{1} males were therefore significantly less successful than *Acp36DE*^{2} and *Acp36DE*$^+$ males in achieving fertilizations when they were the second males to mate. *Acp36DE*^{2} and *Acp36DE*$^+$ males, which do not differ in sperm storage ability (Neubaum & Wolfner 1999), also did not differ significantly in the number of fertilizations achieved as second-mating males.

(ii) *Acp36DE^1 or Acp36DE$^+$ males, lacking sperm*

The displacement ability of spermless *Acp36DE*^{1} (null) males was tested by analysing the number of progeny fathered by first-mating, wild-type males following second matings with spermless *Acp36DE*^{1} or *Acp36DE*$^+$ males. In the two days immediately after remating, females second mated to spermless *Acp36DE*^{1} (null) and *Acp36DE*$^+$ (control) males did not differ significantly in progeny production (three-day rematings, median progeny 74.5 versus 73.0, respectively, Wilcoxon χ^2 approximation = 0.06, $p = 0.79$, four-day rematings, median progeny 96.0 versus 80.0, respectively, $\chi^2 = 0.38$, $p = 0.53$ and five-day rematings, median progeny 36.0 versus 58.0, respectively, $\chi^2 = 0.92$, $p = 0.33$). There were no significant differences in the total progeny (up to six days after remating) produced by females that remated after three, four or five days.

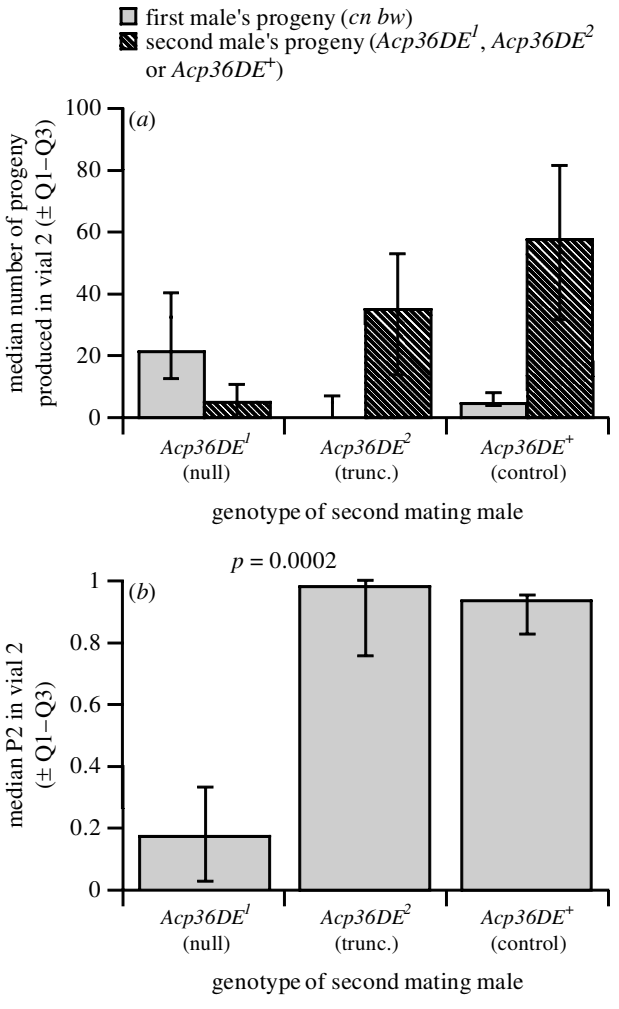

Figure 1. The effect of Acp36DE transfer by second-mating males on second-male progeny production (displacement) in the presence of *Acp36DE*^{1} (null), *Acp36DE*^{2} (truncation) and *Acp36DE*$^{+}$ (control) second-male sperm. (*a*) Median (and interquartile range) first- (dark-grey bars) and second- (hatched bars) male progeny in vial 2 (i.e. from shortly after the second matings until six days later). *cn bw* females were first mated to *cn bw* males and second mated to fertile *Acp36DE*^{1}, *Acp36DE*^{2} or *Acp36DE*$^{+}$ males. (*b*) Median proportion (and interquartile range) of second-male (*Acp36DE*^{1}, *Acp36DE*^{2} or *Acp36DE*$^{+}$) progeny (P_2) produced in vial 2 by the *cn bw* females in (*a*).

Analysis of the combined results showed no significant difference in the total number of progeny produced by females second mated to spermless *Acp36DE*^{1} (median total progeny for all samples = 80) or *Acp36DE*$^{+}$ males (median total progeny = 88) (Wilcoxon χ^2 approximation = 1.40, $p = 0.24$). Second-mating spermless males lacking Acp36DE did not differ from spermless control males in their ability to displace resident first-male sperm.

(b) Acp36DE *and defence* (Acp36DE^{1} (*null*), *Acp36DE*^{2} (*truncation*) *and Acp36DE*$^{+}$ (*control*) *males as first males*)

(i) *Acp36DE*^{1}, *Acp36DE*^{2} *and Acp36DE*$^{+}$ *males, with sperm*

The sperm present in females first mated to *Acp36DE*^{1} (null) males did extremely poorly in sperm defence in all three replicate experiments (females remated after 48, 48 and 24 h for replicates 1, 2 and 3, respectively). What little of the transferred sperm was stored or retained was almost all used before the females were transferred to vial 2 (i.e. by the first few hours after their second matings), resulting in a significant reduction in P_1 compared with first mates of *Acp36DE*^{2} or *Acp36DE*$^{+}$ males. The total number of progeny produced in vial 1 (i.e. from both males, though largely from first males) did not differ between females first mated to *Acp36DE*^{1}, *Acp36DE*^{2} or *Acp36DE*$^{+}$ males (Kruskal–Wallis χ^2 approximation, replicate 1 $\chi^2 = 3.83$, $p = 0.14$, replicate 2 $\chi^2 = 4.42$, $p = 0.1$ and replicate 3 $\chi^2 = 1.59$, $p = 0.44$). There was a short overnight period in which females in vial 1 could lay eggs fertilized by a second male's sperm (i.e. before transfer to vial 2). However, even this was sufficient time for a significant reduction in the number of progeny produced by *Acp36DE*^{1} males relative to *Acp36DE*^{2} or *Acp36DE*$^{+}$ males to become apparent (replicate 1 $\chi^2 = 7.84$, $p = 0.019$, replicate 2 $\chi^2 = 11.42$, $p = 0.0033$ and replicate 3 $\chi^2 = 6.31$, $p = 0.042$) (figure 2). In replicate 3 females were remated after 24 h and would have had more first-male sperm in storage than females in replicates 1 and 2; however, a significant difference in defence due to the presence of Acp36DE was still detectable. Although these experiments were not performed concurrently and, therefore, were subject to differing environmental conditions, the data suggest that remating after 24 h (replicate 3) led to the production of lower first-male progeny than rematings after 48 h (replicates 1 and 2) (see figure 2). Not surprisingly, there was a highly significant reduction in the proportion of first-male progeny (P_1 in vials 2 and 3) produced by females first mated to *Acp36DE*^{1} relative to *Acp36DE*^{2} and *Acp36DE*$^{+}$ males (replicate 1 $\chi^2 = 22.75$, $p < 0.0001$, replicate 2 $\chi^2 = 30.78$, $p < 0.0001$ and replicate 3 $\chi^2 = 35.93$, $p < 0.0001$; data not shown). The total progeny counts in vials 2 and 3 did not differ between groups in any of the replicates (replicate 1 $\chi^2 = 1.266$, $p = 0.53$, replicate 2 $\chi^2 = 1.27$, $p = 0.52$ and replicate 3 $\chi^2 = 4.26$, $p = 0.15$). The results show that males lacking Acp36DE achieved significantly fewer fertilizations as first-mating males compared with males producing the wild-type or truncated Acp36DE protein.

(ii) *Acp36DE*^{1} *and Acp36DE*$^{+}$ *males, lacking sperm*

We analysed the number of progeny fathered by second-mating, wild-type males after initial matings with spermless *Acp36DE*^{1} or *Acp36DE*$^{+}$ males. Females whose rematings with wild-type males were separated by 24 h did not differ significantly in progeny production in any of the four samples ($p = 0.43$, 0.16, 0.07 and 0.30 for females first mating with spermless *Acp36DE*^{1} males and $p = 0.12$, 0.66, 0.97 and 0.63 for females first mating with spermless *Acp36DE*$^{+}$ males). The results were therefore combined for analysis. In the first two days after remating, females first mated to spermless *Acp36DE*^{1} males produced significantly fewer second-male progeny (median = 113.5) than females first mated to spermless *Acp36DE*$^{+}$ males (median = 127.0) (Wilcoxon χ^2 approximation = 6.15, $p = 0.0131$) (figure 3). There were no significant differences in second-male progeny production between the two groups in any other sample.

Females whose rematings with wild-type males were separated by 24 h also did not differ significantly in

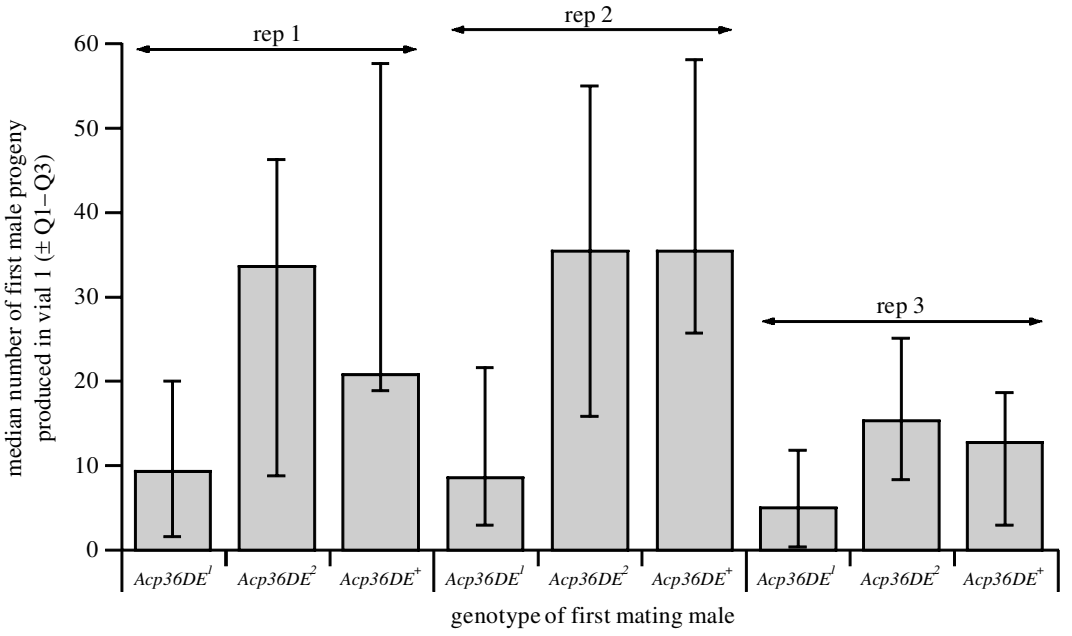

Figure 2. The effect of Acp36DE transfer by first-mating males on first-male progeny production (defence) in the presence of $Acp36DE^1$ (null), $Acp36DE^2$ (truncation) and $Acp36DE^+$ (control) first-male sperm. Median (and interquartile range) first-male progeny in vial 1 (i.e. from first to a few hours after second matings) for three replicate experiments (replicates 1–3). *cn bw* females were first mated to fertile $Acp36DE^1$, $Acp36DE^2$ or $Acp36DE^+$ males and second mated to *cn bw* males (after 48, 48 and 24 h for replicates 1, 2 and 3, respectively).

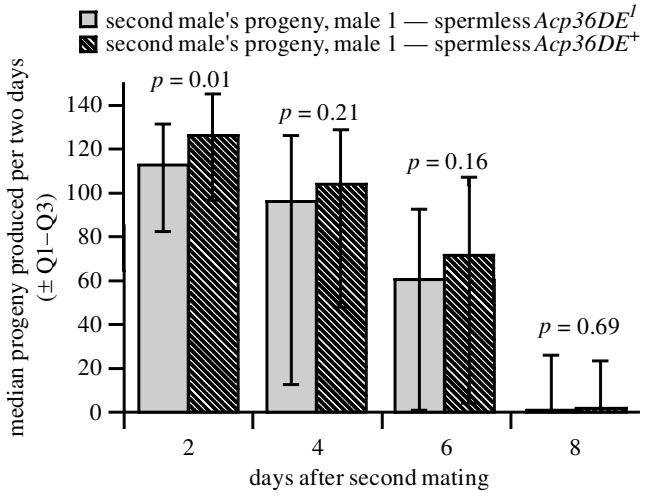

Figure 3. The effect of Acp36DE transfer by first-mating males on second-male progeny production in the absence of sperm provided by $Acp36DE^1$ (null) or $Acp36DE^+$ (control) first males. Median progeny (and interquartile range) per two days against the time in days after second matings. Wild-type females were first mated to irradiated $Acp36DE^1$ (grey bars) or $Acp36DE^+$ (hatched bars) males and second mated to wild-type males.

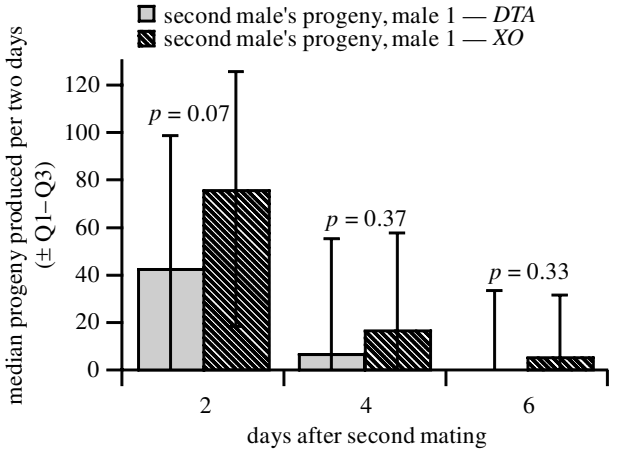

Figure 4. The effect of total main cell Acp transfer by first-mating males on second-male progeny production, in the absence of null Acp and control first-male sperm. Median progeny (and interquartile range) per two days against the time in days after second matings. Wild-type females were first mated to *DTA* (no main cell Acps or sperm, grey bars) or *XO* (Acps but no sperm, hatched bars) males and second mated to wild-type males.

progeny production in any sample in the experiment with *DTA* and *XO* males ($p \geqslant 0.11$ for females first mating with *DTA* males and $p \geqslant 0.42$ for females first mating with *XO* males). The results were again combined for analysis. The number of second-male progeny produced by females first mated to *DTA* (no main cell Acp or sperm transfer) or *XO* males (Acp but no sperm transfer) and injected with Acp70A did not differ significantly for up to six days after the second matings (figure 4). However, in the first two days after second matings, females first mated to *DTA* males produced fewer (median = 41.5) but not significantly fewer offspring after second matings with wild-type males than females first mated to *XO* males (median = 75.0) (Wilcoxon χ^2 approximation = 3.25, $p = 0.07$). The results show that the transfer of Acps by spermless first-mating males resulted in significantly higher progeny production by second-mating males. Taken together our results indicate that Acp36DE from the first male had a facilitatory effect on sperm storage by the second-mating male.

4. DISCUSSION

The Acp36DE protein is essential for the process of sperm storage after single matings (Neubaum & Wolfner 1999). The results of the present study suggest that the effects of Acp36DE also alter the outcome of sperm competition, showing that mutations which affect the process of sperm storage can show up among genes detected in sperm competition assays. Our results show that the previously reported association between *Acp36DE* allelic variation and sperm defence (Clark *et al*. 1995) is likely to be due to variation at the *Acp36DE* locus and not to loci in linkage disequilibrium with it. We also show that effects of Acp36DE transfer from first-mating males can occur with and without sperm co-transfer; therefore sperm transfer is not necessary for the action(s) of Acp36DE to be observed.

Males lacking the Acp36DE protein achieved significantly fewer fertilizations as second males following double matings. For the six days following second matings, females whose second mates did not provide Acp36DE protein produced significantly fewer second-male progeny and had significantly lower P_2-values than mates of males which produced normal, or truncated but functional Acp36DE. Two explanations could account for these findings. $Acp36DE^1$ (null) males may be less successful because fewer of their sperm are stored or retained. Alternatively, Acp36DE could be directly involved in removing the sperm of earlier mating males. The use of irradiated males allowed us to distinguish these alternatives. When irradiated $Acp36DE^1$ or control males were used as the second males, there were no significant differences in the amount of wild-type, first-male sperm displaced. This suggests that Acp36DE exerts its effect on sperm displacement because its lack causes fewer $Acp36DE^1$ males' sperm to be retained for use, not because Acp36DE is involved in sperm displacement *per se*.

These results are not likely to be confounded by differences in egg production following second matings to $Acp36DE^1$ (null), $Acp36DE^2$ (truncation) or $Acp36DE^+$ (control) males. Mates of these males would all have received Acps which stimulate egg production and ovulation (Chen *et al*. 1988; Herndon & Wolfner 1995; Heifetz *et al*. 2000). There were also no significant differences in the ability of spermless $Acp36DE^1$ and spermless $Acp36DE^+$ males (this study, data not shown) or $Acp36DE^1$, $Acp36DE^2$ and $Acp36DE^+$ males which produced sperm (Neubaum & Wolfner 1999) in obtaining rematings with females. The results are therefore not likely to be confounded by any effect on females due to differences in the mating ability of the different males used.

Males lacking Acp36DE also did very poorly in sperm defence. So few of their sperm were stored that very little remained to be used at the time when females remated, resulting in a significant reduction in P_1 for $Acp36DE^1$ males relative to $Acp36DE^2$ or $Acp36DE^+$ males. The large effect of Acp36DE on sperm defence is likely to be accounted for by the fact that so few of their sperm are available to compete. The results from the experiments with irradiated males allowed us to determine whether there was any effect of the Acp36DE protein from first-mating males on sperm storage or use by second-mating males. Females whose first matings were to spermless $Acp36DE^+$ males produced significantly more wild-type progeny than females first mated to spermless $Acp36DE^1$ males in the two days immediately after their second matings to wild-type males. There was also a higher but statistically non-significant ($p = 0.07$) number of progeny produced by females first mated to *XO* males (full Acps but no sperm) compared with *DTA* males (no sperm or main cell Acps including Acp36DE) in the two days following second rematings with wild-type males. The results are not likely to be confounded by fecundity differences. All females received Acps known to affect egg production and laying (Chen *et al*. 1988; Herndon & Wolfner 1995). There were also no differences in the receptivity of females first mated to spermless $Acp36DE^1$ and control males (data not shown). Lack of fecundity-enhancing Acps in the experiment using *XO* and *DTA* males was controlled for by injecting all females with synthetic sex peptide (Acp 70A) (Chen *et al*. 1988).

The results with spermless males therefore suggest a small but significant effect of the Acp36DE protein itself in the absence of sperm co-transfer on progeny production by later-mating males. Transfer of Acp36DE by the first male facilitated sperm storage by the second male. This suggests that lack of Acp36DE from the first male reduces the initial efficiency of sperm storage or use of a later-mating male's sperm and that sperm are not necessary for this effect of Acp36DE to occur. This is a very curious finding for a system in which the ejaculates of different males are expected to be in strong competition. One possibility is that, because the second matings to wild-type males were performed within 24 or 48 h, Acp36DE transferred by the first male persisted long enough to assist in storing the sperm of the second male. Consistent with this idea, although its half-life in the female reproductive tract is not yet known, Acp36DE can localize to its normal oviduct site (at lower efficiency) (Bertram *et al*. 1996) and enter the sperm storage organs (S. Y. Cleland and M. F. Wolfner, unpublished results) without sperm co-transfer. Laboratory and wild females clearly do remate often (e.g. Chapman *et al*. 1994; Harshman & Clark 1998; Imhof *et al*. 1998), but it is unclear whether they routinely do so at a sufficiently high level for first-male facilitation of second-male sperm storage to occur. Another possibility is that Acp36DE acts upon the female nervous system (Arthur *et al*. 1998) in order to prime females for future efficient sperm storage. However, as Acp36DE does not pass outside the genital tract after mating (Bertram *et al*. 1996; Lung & Wolfner 1999), such an effect would have to be very local. The effect of Acp36DE transfer by first-mating males is evident both in the presence and absence of sperm transfer. When co-transferred with sperm, Acp36DE aids in the storage of those sperm. When it enters the female without sperm, Acp36DE can increase the efficiency of storage or use of sperm which are subsequently transferred to the female by a second-mating male. Further investigation is required in order to determine whether the same or separate mechanisms are at work in the presence and absence of sperm.

Clark *et al*. (1995) reported that allelic variation at the *Acp36DE* locus correlated with variation in sperm defence. Our findings indicate that the sperm defence function they detected was probably *Acp36DE* itself and

not simply a locus in linkage disequilibrium with it and that the effect of Acp36DE on sperm competition probably results from its action in sperm storage. The variation in P_1 associated with *Acp36DE* alleles reported by Clark *et al.* (1995) may be attributable to variation in the effects of the different alleles on sperm storage levels. At least some genes uncovered by sperm competition screens may therefore have been detected for their effects on sperm storage rather than for direct roles in the competition between sperm from different males.

The direct association between sperm storage and the outcome of sperm competition that we have highlighted in this study may be the explanation for the lower degree of sperm displacement that is generally reported when second males do not transfer sperm (Scott & Richmond 1990; Harshman & Prout 1994; Price *et al*. 1999; Gilchrist & Partridge 2000). Minimal displacement in the absence of incoming sperm may occur because displaced sperm are re-stored in the absence of new incoming sperm, or Acps such as Acp36DE cannot act upon sperm already in storage. Our results also show that mutations which affect the process of sperm storage can drastically affect the outcome of sperm competition, and highlight the importance of equalizing the number of sperm stored by different marker males. There may be several different effects on aspects of sperm displacement or defence modulated by specific Acps, including Acp36DE, in addition to how each interacts with sperm in determining the overall outcome of sperm competition. Discovering the nature of these mechanisms merits further study and it is clear that further, ever-more carefully controlled experiments are required.

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