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Variation in sex peptide expression in *D. melanogaster*

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Summary

Male *Drosophila melanogaster* transfers many accessory-gland proteins to females during copulation. Sex peptide (SP) is one of these and one of its main effects is to decrease female remating propensity. To date, there has been no investigation of genetic variation in SP-gene expression levels, or if such potential variation directly influences female remating behaviour. We assessed both these possibilities and found significant variation in expression levels of the SP gene across *D. melanogaster* isolines. A non-linear association between SP expression levels and female remating delay suggestive of disruptive selection on expression levels was also documented. Finally, while some isolines were infected with the endosymbiont *Wolbachia*, no association between *Wolbachia* and SP expression level was found.

1. Introduction

In *Drosophila melanogaster*, approximately 112 accessory gland proteins (Acps) are transferred from the male to the female during copulation. These Acps subsequently dramatically alter both female behaviour and physiology (Ram & Wolfner, 2007; Findlay *et al.*, 2008), with functions that include: reducing female remating rate, increasing ovulation and egg laying, and facilitating sperm storage in females thereby increasing male reproductive success (Chen *et al.*, 1988; Neubaum & Wolfner, 1999). Null mutants for *D. melanogaster* Acps such as *Acp70A* (sex peptide (SP)), *Acp26Aa* (ovulin) and *Acp36DE* suffer reduced fertility and/or perform poorly in sperm competition (Herndon & Wolfner, 1995; Neubaum & Wolfner, 1999; Chapman *et al.*, 2001). The genes encoding Acps evolve rapidly both within and between species (Swanson & Vacquier, 2002; Begun & Lindfors, 2005; Mueller *et al.*, 2005; Schully & Hellberg, 2006; Haerty *et al.*, 2007) and variation in their amino acid sequence suggests strong positive selection on these genes (Swanson *et al.*, 2001).

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A major function of the most comprehensively studied Acp, SP, is to induce a female 'refractory' period (reduced acceptance of further matings). This occurs after SP microinjection (Chen *et al.*, 1988) or ectopic expression in females (Aigaki *et al.*, 1991). Additionally, SP reduces female fitness, possibly through the increased production of juvenile hormone (JH) it causes *in vitro* in females' corpora allata (Moshitzky *et al.*, 1996; Wigby & Chapman, 2005; Harshman & Zera, 2007), and because of this, SP has been implicated in sexual conflict, as have seminal proteins generally (Chapman *et al.*, 1995; Eberhard, 1996; Wolfner, 2002; Wigby & Chapman, 2005). The gene encoding SP (*Acp70A*) also shows a strong signal of positive selection and evidence suggests that it is one of the most rapidly evolving Acp genes (Cirera & Aguade, 1997). Sexual selection and/or sexual conflict are likely to be involved in promoting this rapid evolution.

SP is found bound to sperm in females (Peng *et al.*, 2005), and it is then cleaved from the sperm and is thought to interact with both the female genital tract and the nervous system (Ottiger *et al.*, 2000; Yapici *et al.*, 2008). SP's effect on females are prolonged, with SP affecting female behaviour for over 5 days, whereas ovulin, for example, induces shorter term effects on egg laying that last for only one day (Herndon & Wolfner, 1995; Heifetz *et al.*, 2000, 2005;

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Chapman *et al.*, 2003). Other Acps are removed quickly from the female reproductive tract (Monsma *et al.*, 1990; Coleman *et al.*, 1995; Bertram *et al.*, 1996; Ram *et al.*, 2005), but the prolonged occupation of the female reproductive tract by SP is probably because it is bound to sperm (Peng *et al.*, 2005). SP is likely to be a strong determinant of male fitness. This is because female multiple mating is common in *D. melanogaster* and sperm dumping and sperm displacement of the first male's sperm when females remates (Gromko *et al.*, 1984*a*, *b*; Snook & Hosken, 2004) mean the last male to mate often sires ~80% of subsequent offspring. Therefore, a male's ability to prevent females from remating will be an important male fitness component and SP affects the duration of this delay (Fiumera *et al.*, 2007).

Variation in transcript levels of protein-coding genes is thought to be responsible for many of the phenotypic differences within and between populations of *D. melanogaster*, including sexual dimorphism (Baker *et al.*, 2007), exemplifying how important transcriptional regulation can be. Additionally, studies of variation in *Acp70A* gene sequence show several polymorphisms either within or just upstream of the *Acp70A* coding region (Fiumera *et al.*, 2007). Such polymorphisms in *Acp70A* gene sequence occur in natural populations, yet the natural variation in expression levels of *Acp70A* has not been previously examined, nor is it known if variation in expression levels leads to variation in female responses, such as delaying remating.

Endosymbionts are also known to have drastic effects on host sexual behaviour by manipulating their reproductive physiology and/or behaviour (Folstad & Karter, 1992; Min & Benzer, 1997; Champion de Crespigny *et al.*, 2006; Negri *et al.*, 2008). One such obligate intracellular organism is the bacterium *Wolbachia* (Jeyaprakash & Hoy, 2000), which is widespread in insects (Werren *et al.*, 1995) and occurs at frequencies as high as 30–75% in both wild and laboratory populations of *D. melanogaster* (Corby-Harris *et al.*, 2007). *Wolbachia* is present in almost all *Drosophila* tissues with highest infection levels in the ovaries of females (Dobson *et al.*, 1999), where they infect the eggs and are transmitted to any offspring subsequently produced (reviewed in Tram *et al.*, 2003). Crosses between infected males and uninfected females cause reduced egg-hatching success due to cytoplasmic incompatibility (CI) (Werren, 1997). In *Nasonia* and *Drosophila*, CI appears to occur because the two sister sets of chromosomes do not align synchronously at meiosis (Tram & Sullivan, 2002; Tram *et al.*, 2003), but the molecular mechanism by which *Wolbachia* induces the CI phenotype is still unknown. Although evidence for fecundity costs associated with *Wolbachia* infection is inconsistent (Hoffmann *et al.*, 1994; Min & Benzer, 1997; McGraw *et al.*, 2002; Weeks *et al.*, 2007), in *Drosophila simulans Wolbachia* infection causes reduced sperm production in males (Snook *et al.*, 2000) and poor competitive ability of sperm when competing with other males' sperm for fertilization of ova within females (Champion de Crespigny *et al.*, 2006). Fewer sperm and low sperm competitive ability may generate selection on other ejaculate components to compensate for these detrimental effects. One target of compensating selection could be SP as this influences females' remating propensity and hence reduces sperm competition risk.

Here, we test for variation in *Acp70A* expression levels in 15 isofemale lines of *D. melanogaster* and then assess the effects this variation has on the duration of the female refractory period, a key target of SP, and an important male fitness component. We also test for the effects of *Wolbachia* on *Acp70A* gene expression patterns as the negative impact of this parasite on other male fitness components (e.g. sperm number) has the potential to select for compensatory increases in SP production.

2. Materials and methods

(i) *Rearing conditions*

D. melanogaster isofemale lines were collected by Trudy Mackay in North Carolina in 2004, donated to us by Frank Jiggins and continually maintained by full sib mating. They arrived in our lab in February 2007 and were reared in 7·5×2·5 cm glass vials with approximately 50 individuals per vial. Vials containing flies were kept at 25 °C on a 12:12 light:dark light cycle with 15 ml standard food mix (10 g agarose, 85 g granulated sugar, 60 g maize, 10 g yeast, 1 litre deionized H_2O and 1 g nipagin). Before the experiment six individuals from each line were diagnosed for *Wolbachia* infection by PCR following Snook *et al.* (2000) after DNA extraction using EDNA kits (Fisher Biotech). Due to the isoline status of the flies and high transmission fidelity of *Wolbachia* (Hoffmann *et al.*, 1990) this was taken as an indication that all flies are either infected or uninfected within lines.

To generate experimental flies reared under standardized conditions, populations laid eggs on 2×1 cm laying caps of food mix in 9×2·5 cm universal tubes for 24 h. Low-density vials were set-up with 40 eggs placed on approximately 7 ml standard food mix vials to reduce larval competition. Upon emergence virgin adults were collected under CO_2 anaesthesia and sexed on ice every 8–12 h. Males were placed individually in standard food vials and females were discarded. Five-day-old males were subsequently frozen in liquid nitrogen and stored at −80 °C.

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(ii) Acp70A *expression*

RNA was extracted from 2 to 5 males per isofemale line using Tri reagent (Sigma) and treated with DNase (Sigma). PCR was used to confirm complete DNA removal using *Acp70A* specific primers FP: 5′-CGTTTGCGTACTCGGCTTGGTC, RP 5′-CCCC-AAATTAAGACGGCACCACT. (PCR cycle: 95 °C for 3 min, followed by 39 cycles of 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 5 min). 10 μl reactions were used containing 1 μl RNA sample, 1 μl 10 pM primers and 7 μl $1{\cdot}1\times$ ReddyMix™ (ABgene). 5 μl of sample was run on 1·4% agarose gel at 120 V for 30 min and viewed under a UV lamp.

Quantitative reverse transcriptase PCR (Q-RTPCR) was carried out using a DNA engine Opticon 2 with FullVelocity® SYBR® Green one-step Q-RTPCR Reagents (Stratagene). The housekeeping gene RP49 (primers FP: 5′-ATCCGCCCAGCATACAG, RP: 5′-TTCGACCAGGTTACAAGAA) was used to normalize overall expression levels and *Acp70A* primers used to quantify expression levels (5′-GAATGGC-CGTGGAATAGGAA, RP 5′-GGCACCACTTAT-CACGAGGATT (Chapman *et al.*, 2003)). Standard curves for both primer pairs were established using serial dilutions of total RNA concentration across four orders of magnitude (*Acp70A* efficiency: 93·7%, RP49 efficiency: 104·5%). A sub-sample of individual flies were run twice on different Q-RTPCR runs and found to be highly repeatable across PCRs (regression of PCR1 on PCR2 $n=52$, mean $r=0{\cdot}70$ and mean $\beta>0{\cdot}65$, $P<0{\cdot}0001$).

Relative *Acp70A* expression was calculated by taking the difference between the cycle threshold values (Cts) for the housekeeping gene and the *Acp70A* gene. All reactions were carried out in triplicate and a melt curve produced after each run to check priming specificity. Any Cts that were not within 0·5 cycles of the other triplicates were removed from final analysis. To normalize the data, the largest relative expression level was taken from an individual fly and given the value 1. All other values were converted to a value relative to this.

Log transformed Pfaffle (Pfaffl, 2001) and ΔΔCt (Livak & Schmittgen, 2001) were used for analysis and results were essentially identical. As a result only ΔΔCt data will be presented here. Data and residuals were normally distributed ($Z=1{\cdot}141$, $P=0{\cdot}148$, $Z=1{\cdot}341$, $P=0{\cdot}055$ respectively). Analyses were conducted using SPSS (SPSS Inc. Version 11 for Mac).

To test for effects on differences in *Acp70A* gene expression on females, we conducted a separate experiment assessing the duration of the female mating delay. Here males from nine of the experimental lines were collected as virgins and aged as before. Virgin females from a non-related isoline were collected and housed in vials containing up to 40 individuals. Between 1 and 11-five-day-old virgin males from each line were then mated to 3-day-old virgin females of the non-related line. All females were then housed individually and after 48 h they were exposed to virgin males of another non-related line every day for 4 h until all females had remated.

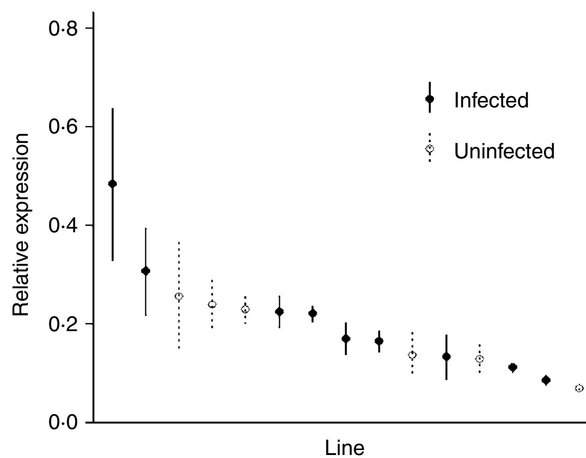

Fig. 1. *Acp70A* RNA levels differ across field-collected lines of *D. melanogaster*. Graph shows relative *Acp70A* expression for 15 lines of isoline 5-day-old virgin males either infected (full circle, full line) or uninfected (open circle, dotted line) with *Wolbachia*. Data points represent line means ± SE and are plotted from highest (left) to lowest (right) for visual purposes.

3. Results

We used isofemale lines of *D. melanogaster* to investigate natural genetic variation in *Acp70A* expression levels. Q-RTPCR was used to measure the transcript levels of *Acp70A* in individual males from each line. We also examined if *Wolbachia* infection affects expression of *Acp70A*. Using general linear mixed models (GLMMs) with isoline nested within *Wolbachia* infection status (infected *v.* uninfected), we found a significant effect of isoline, indicating genetic variation in *Acp70A* transcript levels across the 15 isolines ($F_{13,43}=2{\cdot}64$, $P<0{\cdot}01$, Fig. 1), with a 5-fold difference in mean *Acp70A* expression levels across lines. In contrast, *Wolbachia* infection status was not associated with differences in *Acp70A* expression levels ($F_{1,14}=0{\cdot}34$, $P=0{\cdot}57$).

Ordinary least-squares regression was used to test for an association between *Acp70A* transcript levels and female remating rates across isolines. Initial viewing of this association suggested a polynomial relationship and a polynomial regression revealed a significant polynomial association between mean *Acp70A* expression levels and median time for remating by females mated to males from each line ($n=9$ lines, $r=0{\cdot}81$, $F_{2,6}=5{\cdot}64$, $P=0{\cdot}04$, Fig. 2). The model lost significant explanatory power if either the

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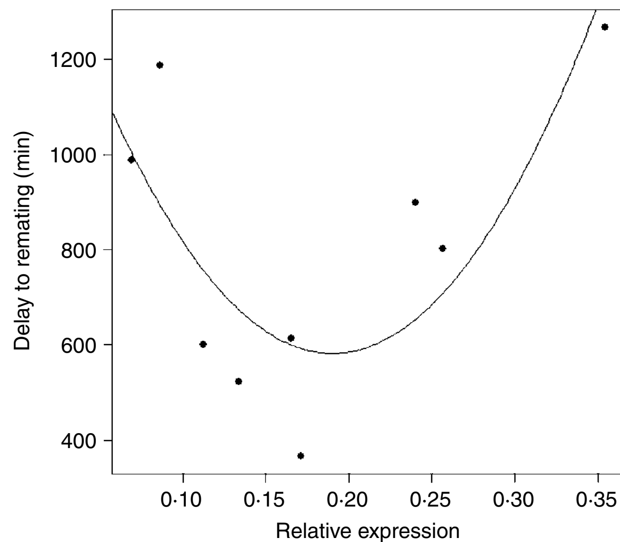

Fig. 2. Polynomial regression shows a significant quadratic association between relative *Acp70A* expression levels of males and the time taken for females to remate to a standard male. Interestingly, males expressing *Acp70A* at high and low levels are associated with longer refractory periods in females than males expressing intermediate levels of *Acp70A*, suggesting disruptive selection for *Acp70A* expression. Means of 1–11 males per line.

linear or the quadratic term was removed so both were retained (linear term: $t=-2{\cdot}8$, $P=0{\cdot}03$, quadratic term: $t=3{\cdot}1$, $P=0{\cdot}02$).

4. Discussion

Our major findings were that there was significant genetic variation in *Acp70A* transcript levels and that this variation had a non-linear effect on female remating. Previously Fiumera *et al.* (2005, 2007) have shown that DNA sequence variation in some Acp genes is associated with male fitness and that *Acp70A* polymorphisms are associated with varying refractory periods in females. Sequence polymorphisms may be one mechanism by which transcriptional variation occurs. Here, we show that genetic variation also exists in expression levels of a particular Acp gene, *Acp70A* with approximately 5-fold differences apparent across isolines. This variation is obviously a prerequisite for the evolution of *Acp70A* expression differences, and variation could be maintained by condition dependence, as proposed for other sexually selected traits (Rowe & Houle, 1996). This possibility remains to be tested.

In *D. melanogaster*, male Acp stocks become depleted after mating (Monsma *et al.*, 1990; Linklater *et al.*, 2007) and are a limiting factor to mating success in another non-Drosophilid fly species (Rogers *et al.*, 2005). This suggests that depletion of Acps is likely to directly influence male fitness (Hihara, 1981), although the full benefits of having larger stocks of SP may only become apparent after repeated mating. High *Acp70A* expression levels may enhance a male's ability to replenish accessory gland stores of SP more quickly or directly influence male SP-store volume. This remains to be established.

While genetic variation is needed for SP evolution, if there is no phenotypic variation in its effects, there will be no selection on that variation. To that end, SP induces a refractory period in females for up to 5 days post copulation and here we documented a significant association between *Acp70A* expression levels and the time taken for females to remate after a single copulation with a male from an experimental line. This association was non-linear, with a longer delay for low- and high-expression levels of *Acp70A*. Both the linear and quadratic effects were significant in our analysis, with the negative linear term evidently explaining some proportion of the variation in the left had section of Fig. 2 (when relative expression was less than *c.* 0·2). Although we have not investigated all potential effects, this first assessment suggests that there is disruptive selection acting on *Acp70A* expression as males with intermediate *Acp70A* expression levels suffered a relative cost in terms of female propensity to remate. As yet, it is not known whether variation in *Acp70A* expression between isolines directly relates to difference in the amount of SP transferred to females at mating, and/or whether there is variation in the 'potency' of the transferred SP as a suppressor of female receptivity. Precisely how this relates to other potential SP effects is unknown, as are associations between this and other Acps, but it appears that there is genetic variation in and selection on expression levels of *Acp70A*.

We find no effect of *Wolbachia* infection on expression levels of *Acp70A*. We acknowledge that with these sample sizes our power is relatively limited, but at this point in time, we must conclude there is no obvious interaction between *Wolbachia* and *Acp70A* expression in virgin males. Similarly, in *D. simulans*, Snook *et al.* (2000) found no difference in the amount of other Acp proteins (ovulin and Acp36DE) transferred to females by infected and uninfected males. Sperm production, however, was lower in infected males (Snook *et al.*, 2000). This sperm deficit is exacerbated as males mate repeatedly, resulting in reduced sperm competitive ability (Champion de Crespigny & Wedell, 2006). Similarly, *Wolbachia* infection may only affect *Acp70A* expression after several matings when either sperm and/or Acp stocks are depleted. Alternatively, there may be less need for *Wolbachia*-infected males to produce SP because there are fewer sperm to which it can bind. Studies examining the plasticity of SP binding to sperm are needed to test these ideas. Additionally it is as yet unknown whether *Wolbachia* infection affects

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D. melanogaster sperm production in the same manner as *D. simulans*.

In conclusion, we have shown natural variation in *Acp70A* expression in field-collected isolines of *D. melanogaster* corresponding to a 5-fold difference in RNA levels. With this variation in *Acp70A* expression levels we also expected to see phenotypic differences in its effect. However, the observed association was not a simple linear relationship. Instead we found evidence for disruptive selection on *Acp70A* expression levels through its effects on female remating delays. How this relates to other Acps and male fitness components remains to be investigated.

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