Larval rearing environment affects several post-copulatory traits in
Drosophila melanogaster

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In Drosophila melanogaster, accessory gland proteins (Acps) that a male transfers during mating affect his reproductive success by altering the female's behaviour and physiology. To test the role of male condition in the expression of Acps, we manipulated the pre-adult environment and examined adult males for relative transcript abundance of nine Acps, and for post-copulatory traits that Acps influence. Larval culture density had no effect on any measured trait. Larval nutrient availability impacted the number of sperm transferred and stored, the male's ability to induce refractoriness in his mate, but relative transcript abundance of only a single Acp (Acp36DE). Reduced male body size due to low yeast levels affected sperm competition. Our data indicate that some female-mediated post-copulatory traits (induced refractoriness and sperm transfer and storage) might be influenced by the male's developmental environment, while relative expression of most Acps and some traits they influence (P1) are not.

Keywords: accessory gland proteins; sperm competition; sperm storage; transcript levels; body size

1. INTRODUCTION

In Drosophila melanogaster, accessory gland proteins (Acps) that males transfer to females during mating impact traits associated with post-copulatory sexual selection by modulating the behaviour and physiology of mated females. Acps regulate egg laying and oviposition, affect sperm storage, reduce a female's receptivity to remating and associate with variation in sperm competition phenotypes (reviewed in Wolfner 2002; Kubli 2003; Wolfner et al. 2005; Wong & Wolfner 2006).

If Acps are more costly to produce than other non-sexually selected traits, we predicted that they may exhibit a relative reduction in expression under stress

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2. MATERIAL AND METHODS

(a) Rearing conditions

Three-day-old virgin males were frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted using Trizol (Invitrogen), DNase-treated (Roche) and cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) primed with oligo d(T)18. Six extractions were conducted for each condition and two replicates were performed for each condition PCR reactions (qRT-PCR) were completed for each cDNA extraction.

Relative Acp transcript abundance was measured using qRT-PCR on an ABI Prism 7000 according to the manufacturer’s protocols for SYBR Green. Detailed methods and primer sequences are available (except for Acp29AB, whose primers were TGGAGTT-GAATAAGGCCAGATG (forward) and GCCATTTTCACGAGTTTGG (reverse)) are described in Fiumera et al. (2005). To control for body size and efficiencies of RNA extraction and cDNA synthesis, we neglected each Acp’s inverse size marker (1/CT) from the qRT-PCR against its corresponding ‘1/CT’ measure for an internal control gene, RPL32. Analysis of variance (ANOVA) (using regression residuals) was used to test for treatment effects with sequential Bonferroni corrections (Rice 1989). Using raw CT values provided similar results (data not shown).

(b) Relative Acp transcript abundance

Relative Acp transcript abundance was measured using qRT-PCR on an ABI Prism 7000 according to the manufacturer’s protocols for SYBR Green. Detailed methods and primer sequences are available (except for Acp29AB, whose primers were TGGAGTT-GAATAAGGCCAGATG (forward) and GCCATTTTCACGAGTTTGG (reverse)) are described in Fiumera et al. (2005). To control for body size and efficiencies of RNA extraction and cDNA synthesis, we neglected each Acp’s inverse size marker (1/CT) from the qRT-PCR against its corresponding ‘1/CT’ measure for an internal control gene, RPL32. Analysis of variance (ANOVA) (using regression residuals) was used to test for treatment effects with sequential Bonferroni corrections (Rice 1989). Using raw CT values provided similar results (data not shown).

(c) Sperm competition phenotypes

Only the ‘defense’ components of sperm competitive ability were measured, because the proportion of offspring sired by inbred marker males when they were the second male to mate with a doubly mated female (P2) was nearly 100% across different larval environments in preliminary experiments (data not shown). Refractoriness is the proportion of females mated to experimental or control males that do not remate with a tester male. P1 is the proportion of offspring sired by the experimental or control males when they are the first males to mate with a doubly mated female. Fecundity is the total number of offspring produced by each doubly mated female. Productivity was assessed by singly mating each female to males from the yeast treatments only and tallying progeny over 14 days. Approximately 30 females were used for each treatment.

One-way ANOVA was used to test for an effect of the larval environment on P1 (arcsine-square-root transformed), female fecundity (square root transformed) and productivity (squared). Permutation tests based on chi-squared statistics (MatLab) were used to test for significant heterogeneity among treatments in female refractoriness. Sequential Bonferroni corrections (Rice 1989) were applied.
(d) **Sperm transfer and storage**

Three-day-old virgin marker females (on bw) were mated to three- or four-day-old males reared on 100, 50 or 10% yeast and frozen in liquid nitrogen immediately after copulation (to estimate sperm transferred) or after 8 hours (to estimate sperm stored) and stored at -80°C. Uteri (for sperm transfer) and seminal receptacles (for stored sperm) were dissected in 50% acetic acid and incubated in orcein stain (2% orcein and 0.25% carmine dissolved in 60% acetic acid) for 1 hour and then mounted on a slide containing a drop of acetic acid, covered with a cover slip and sealed with nail polish. Sperm were counted under 100× magnification light microscopy. Data were analysed using ANOVA.

(e) **Body size**

Four-day-old male flies were dried at 60°C for 24 hours in an oven and weighed on a Sartorius microbalance. We used linear regression to examine effects of body size on treatments. For PI', refractoriness, sperm transfer and storage, we compared treatment means of body weight versus treatment means of each trait and used the residuals in ANOVA if r² was significant (p≤0.05).

3. RESULTS

(a) **Relative Acp transcript abundance**

*RPL32* transcript abundance did not differ between treatments, but was positively correlated with transcript abundance of all nine Acp genes (r²=0.54–0.97). Larval culture density had no effect on the relative transcript abundance of any of the tested Acps, while larval nutrient availability affected the relative transcript abundance of *Acp36DE* (F4,25=4.658, p=0.006). Separate one-way ANOVAs revealed that larval dextrose availability had no effect on the relative transcript abundance of *Acp36DE*, but males reared on 50% yeast had reduced relative levels of *Acp36DE* transcript when compared with controls (F2,15 = 8.0645, p=0.004; figure 1a).

(b) **Sperm competition phenotypes**

Larval culture density did not affect any of the measured sperm competition phenotypes (*N=56 females; p>0.30 for all phenotypes*). In contrast, nutrient availability during larval life affected PI' (F4,94=3.19; p=0.017; marginally significant after sequential Bonferroni correction) and female refractoriness (*N=119 females in five treatments; p=0.015 from permutation test). Larval nutrition did not affect female fecundity (F4,90=1.00; p=0.41) nor did larval yeast availability affect female productivity (F2,75 = 1.64; p=0.20). Separate one-way ANOVAs revealed that dextrose levels in larval medium had no effect on PI' (F2,46=1.30; p=0.282), but yeast levels did (F2,62=3.89; p=0.026; significant after Bonferroni correction). Males reared on 10% yeast sired a lower proportion of offspring when compared with controls (Tukey test, p=0.023; figure 1b). Pairwise comparisons among all treatments revealed that female refractoriness was marginally elevated when mates to males reared on 10% dextrose (0.46±0.10 s.e. versus 0.17±0.08 s.e.; p=0.03; figure 1c) and was marginally reduced when mates to males reared on 10% yeast (0.04±0.04 s.e. versus 0.17±0.08 s.e.; p=0.05; figure 1c) when compared with controls. In addition, significant differences in refractoriness were detected between females mated to males reared on 10% dextrose versus males reared on either 10% yeast (0.46±0.10 s.e. versus 0.04±0.04 s.e.;

![Figure 1](image-url). Means and standard errors of the effects of larval rearing conditions on (a) relative *Acp36DE* transcript abundance given as the residual 1/CT value regressed against a control gene, *RPL32*, (b) PI' and (c) relative refractoriness. Larval rearing conditions are shown on the x-axis. Percentage of yeast and dextrose is given relative to the control (100%). Density is given as the number of larvae per vial.

p=0.0007; figure 1c) or 50% yeast (0.46±0.10 s.e. versus 0.08±0.05 s.e.; p=0.001; figure 1c).

(c) **Sperm transfer and storage**

Larval yeast availability significantly affected the amount of sperm males transferred to females (F2,29=22.43, p<0.0001; figure 2a). Males reared on 10% yeast transferred fewer sperm than control males or those reared on 50% yeast (Tukey test, p<0.0001 for both cases; figure 2a). Larval yeast availability also significantly affected the number of sperm stored in the male's seminal receptacles (F2,34=5.5423, p = 0.0083; figure 2b). Females retained fewer sperm when mated to males reared on 10% yeast when compared with males reared on 50% yeast, but not control males (Tukey test, p=0.0113; figure 2b).

(d) **Body size**

Larval rearing conditions significantly affected male body size (F6,133=18.61; p<0.001; see table 1 in the
We find little evidence that relative Acp transcript abundance depends on the male’s larval rearing environment. Larval yeast availability affected the relative transcript levels of only one of the nine tested Acps (Acp36DE). The correlation between the effects of larval environment and relative Acp36DE transcript levels was not linear as expected, but only reduced when males were reared on 50% yeast. Because females require Acp36DE for normal sperm storage (reviewed in Bloch Qazi & Wolfner 2003), we explored whether sperm storage was decreased when females mated to these males. We found that females mated to 50% yeast males store significantly more sperm than 10% yeast males and about the same numbers as control males. Thus, the decrease that we observed in relative Acp36DE transcript abundance when males were reared on 50% yeast does not suggest that these males transfer less Acp36DE to the female. We can currently offer no explanation as to why relative Acp36DE transcript levels were lower when yeast levels were only moderately reduced.

We note that while relative Acp transcript levels were unchanged in most treatments, protein levels may be affected. Thus, it will be of future interest to determine if the larval rearing environment affects the amount of Acp protein transferred to the female.

In summary, our experiments suggest that males’ abilities to sequester and allocate resources in response to the larval environment may ultimately affect variation in several traits involved in post-copulatory sexual selection.

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4. DISCUSSION

Our manipulations of the male’s larval rearing environment led to differences in male body size across treatments with 10% yeast males being the smallest. We found that effects on PI’ were probably a direct result of these differences in body size. Our results, as well as a report that studied other traits (Amitin & Pitnick 2006), suggest that in D. melanogaster, male body size plays a fundamental role in determining the outcome of many post-copulatory processes.

Interestingly, two post-copulatory traits that were reduced when males were reared on 10% yeast (female refractoriness and the number of sperm that females stored) were not correlated with male body size. Of the phenotypes that we measured, these two represent processes over which the female may have the most control (Eberhard 1996) and are physiologically intertwined. Females who have fewer sperm in storage are more likely to remate (reviewed in Bloch Qazi et al. 2003).

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