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PERCEIVED SPERM COMPETITION INTENSITY INFLUENCES SEMINAL FLUID PROTEIN PRODUCTION PRIOR TO COURTSHIP AND MATING

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Sperm competition is a potent postcopulatory selective force where sperm from rival males compete to fertilize a limited set of ova. Considering that sperm production is costly, we expect males to strategically allocate sperm in accordance with the level of competition. Accordingly, previous work has examined a male's strategic allocation in terms of sperm number. However, the seminal fluid proteins (Sfps) transferred along with sperm may also play a crucial role in competition. Surprisingly, the strategic allocation of Sfps has remained largely unexplored. Using *Drosophila melanogaster*, we examined the expression of three seminal fluid and four spermatogenesis genes in response to perceived sperm competition intensity by manipulating male density in a pre-mating and courtship environment. In the pre-mating environment, we found that males modified Sfp ratios by reducing the production of two spfs when potential rivals were present, while one Sfp and all spermatogenesis genes remained unaltered. In the courtship environment, males did not modify spermatogenesis or Sfp production in response to either rival males or female presence. Our data suggest that perceived competition in the pre-mating environment places a significant influence on Sfp allocation, which may be a general trend in promiscuous animal systems with internal fertilization.

KEY WORDS: **Acps, *Drosophila melanogaster*, ejaculate, gene expression, spermatogenesis.**

Over the past several decades, the realization that selection on male reproductive success extends beyond copulation has redefined our view of sexual selection (Simmons 2001; Eberhard and Cordero 2003). Postcopulatory sexual selection has the power to shape numerous aspects of biology, from the evolution of anisogamy to reproductive behavior. Perhaps the most potent postcopulatory selective force is sperm competition, where sperm from rival males compete to fertilize a given set of ova (Parker 1998). Considering that sperm production is costly (Pitnick and Markow 1994; Kaitala and Wiklund 1995; Prowse and Partridge 1997), we expect males to strategically allocate their ejaculate in accordance with the level of competition (Dewsbury 1982). Numerous theoretical models have been proposed to predict optimal ejaculate allocation strategies in response to sperm competition risk (i.e., the population's average probability that a given female will remate; Parker et al. 1997) or sperm competition intensity (i.e., the number of competing ejaculates at the time of fertilization; Parker et al. 1996). Despite the differences in these models with regard to their scope (e.g., within or across species) and mathematical detail, both are generally tested by empirically assessing sperm number. Numerous other male ejaculate traits, however, may influence the outcome of competition (Pitnick and Markow 1994; Snook 2005).

In animals with internal fertilization, seminal fluid proteins (Sfps) make up a significant portion of the ejaculate, and may play a crucial role in sperm competition or its avoidance (Chapman 2001). In insects, Sfps can form mating plugs (Baer et al. 2000), increase a female's latency to remate (Chapman et al. 2003; Liu and Kubli 2003), increase egg laying rate (Heifetz et al. 2000; Chapman et al. 2003; Liu and Kubli 2003), modify sperm storage (Neubaum and Wolfner 1999), as well as alter female lifespan (Chapman et al. 1995; Wigby and Chapman 2005). Given the potential importance of these proteins in competition, a recent theoretical work suggested that both sperm and Sfps should be allocated strategically whenever females differentially use sperm (e.g., last male sperm precedence; Cameron et al. 2007). The specific allocation predictions, however, depended on Sfp function (e.g., fecundity enhancing or male fertility enhancing proteins). Surprisingly, the strategic allocation of these proteins in response to sperm competition has remained largely unexplored both theoretically and empirically (but see Wigby et al. 2009).

For most animal systems it is generally assumed that sexually active males continually produce and store sperm and Sfps. This continued production can be divided into three reproductive episodes including pre-mating, courtship, and mating. Within each episode, males may actively assess a dynamic reproductive environment (e.g., operational sex ratio, occurrence of previously mated females, presence of rival males, rival male quality, and potential mate quality) and modify the total production of Sfps and sperm, as well as the ratio of specific Sfps, to create an optimized ejaculate "cocktail." The extent of production modification in a given episode may depend largely on episode duration. For instance, we may expect the largest modifications in production in the hours or days leading up to courtship (the pre-mating episode), with relatively little change in production during the courtship or mating episode (animal courtship and mating is often brief). In contrast, males may greatly modify ejaculate donation during the mating episode by adjusting the contraction of smooth muscle surrounding Sfp and sperm stores or by altering the duration of copulation (Bretman et al. 2009). However, the donation of variable Sfp ratios in direct response to a variable mating environment may be impossible, unless males possess separate accessory glands that hold different proteins or protein ratios prior to mating, or if significant changes in protein production rapidly occur once mating begins.

Here we examine the production (not the donation) of seminal fluid and spermatogenesis proteins during the pre-mating and courtship episodes in response to perceived sperm competition intensity in the fruit fly, *Drosophila melanogaster.* In this system, males mate opportunistically; however, the production of seminal fluid protein is limiting (Sirot et al. 2009). Furthermore, natural population variation in these proteins is tightly linked to variation in sperm competitive ability (Fiumera et al. 2005, 2007). Thus, the opportunity for strategic allocation of seminal fluid protein and sperm exists. To examine this possibility, we manipulated male density during the pre-mating and courtship episodes and estimated mRNA levels. We found that the presence of rival males influenced the production of seminal fluid proteins in the pre-mating episode, but not in the courtship episode. In addition, spermatogenesis protein production was unaltered in both episodes.

Methods

FLY STOCKS AND MAINTENANCE

The experimental stock used in our study was derived from 34 wild caught gravid females collected near Orlando, Florida, in the summer of 2008. This stock was maintained as a large outbred population (approximately 300 individuals per generation) for approximately 22 generations in 30 cm^3 cages on a standard cornmeal-yeast medium. Two generations prior to the experiment, a sub population of randomly chosen individuals was maintained in 30 mL vials at 24°C with a 12:12 L:D photoperiod.

EXPERIMENTAL DESIGN

To estimate ejaculate allocation in response to rival male presence during premating, low intensity (1 male per vial) and high intensity (4 males per vial) sperm competition treatments were established. These densities are common in both theoretical and empirical sperm competition studies (Parker et al. 1996; Aspbury 2007). Experimental flies were separated by sex under light CO_2 anesthesia within 4 h of adult eclosion to insure virginity. Male flies were randomly assigned to either treatment, creating a total of 192 low intensity vials and 48 high intensity vials (females were discarded). At 6, 24, 48, and 72 h after adult eclosion, a subset of vials from both treatments were randomly chosen and assayed for mRNA levels. For each time point, 48 low intensity male vials were chosen to create 6 mRNA samples. Similarly, 12 high intensity male vials were chosen and mixed to create 6 mRNA samples. Additionally, 6 samples were collected at adult eclosion (time point 0) prior to the assignment of any treatment. Each sample contained 8 flies placed in Trizol, which was immediately homogenized and stored at −80°C for future RNA extraction.

To estimate rapid changes in ejaculate allocation during courtship, virgin males and females were collected as before and placed into single density vials. Three days after adult eclosion, a subset of males and females were paired and allowed to mate for 1 h; females who did not mate were discarded, as were all males used for mating. Four days after adult eclosion, the remaining virgin males were randomly assigned to one of four treatments that simulated sperm competition intensity, including a low intensity

(1 male per vial), medium intensity (2 males per vial), high intensity (4 males per vial), and low intensity control (1 male per vial; no courtship) treatment. These treatments were established by gently combing the single density vials into a new treatment vial without anesthesia (single intensity treatments were transferred to new vials for consistency). Treatment vials were allowed to settle for 3 m before the addition of a single previously mated female (newly mated females ensure a low probability of mating during the courtship phase; Gromko and Pyle 1978; personal observation). Males were allowed to court the female for 8 m, after which time the vials were placed under light CO_2 anesthesia and the females discarded. Any vials that contained a mating pair were also discarded from the experiment (<3%). The 8 m time point was chosen considering that previous work in *D. melanogaster* indicated that such rapid changes in the social environment can significantly influence gene regulation (Carney 2007). The anesthetized males were then mixed within their treatments to create 6 mRNA samples, each containing 8 males placed in Trizol (Invitrogen). As before, samples were immediately homogenized and stored at −80°C for future RNA extraction. In total, 192 males were equally partitioned across the four treatments.

GENES AND GENE EXPRESSION

We examined mRNA transcription levels for three well characterized seminal fluid proteins including Acp70A (CG17673), Acp26Aa (CG8982), and Acp62f (CG1262). Previous work in *D. melanogaster* indicates that Acp70A influences a female's latency to remate (Chapman et al. 2003; Liu and Kubli 2003); both Acp70A and Acp26Aa influence female oviposition rate (Wolfner et al. 1997; Heifetz et al. 2000; Chapman et al. 2003; Liu and Kubli 2003); and Acp62f influences a male's defensive sperm competitive ability (Mueller et al. 2008). In addition, we assayed two genes (cookie monster [CG13493] and vismay [CG8821]) associated with the early stages of spermatogenesis, and two genes (don juan [CG1980] and gonadal [CG33756]) associated with the final stages of spermatogenesis (Fuller 1998; Ayyar et al. 2003; Wang and Mann 2003; Catron and Noor 2008).

Expression levels for both seminal fluid and spermatogenesis genes were estimated by first isolating mRNA from our samples using a standard chloroform/isopropanol extraction with a Turbo DNase (Ambion, Austin, TX) treatment to remove residual genomic DNA. We then reverse transcribed the samples using the Invitrogen Superscript III kit (Invitrogen, Carlsbad, CA). The resulting cDNA was maintained at −80°C, until real-time qPCR could be performed. Gene expression quantification was accomplished using a Bio-Rad MyIQ single-color optical detection system and the SybrGreen Supermix (Bio-Rad, Hercules, CA). All primers were designed from the published sequence (available at www.Flybase.org) using PRIMER3 (version 0.4.0) and NETPRIMER software. Only primers that exhibited high PCR efficiency (higher than 95%) and no spurious amplification were used. To test primer specificity, we blasted each primer pair against the Drosophila genome (www.ensembl.org), as well as performed a melt curve and agarose gel separation on the PCR product.

STATISTICAL ANALYSIS

For the pre-mating episode, we estimated gene expression ($2^{\Delta Ct}$) by scaling the target gene's cycle threshold value (Ct) to the Ct value of an endogenous control gene (actin-5) for each sample (i.e., $\Delta Ct_i = Ct[control]_i - Ct[target]_i$, where i represents the sample [modified from Livak and Schmittgen 2001]). For the courtship experiment, we estimated gene expression ($2^{\Delta\Delta Ct}$) by first scaling the experimental ΔCt to the ΔCt of the control treatment (i.e., $\Delta\Delta Ct = \Delta Ct(experimental)/\Delta Ct(control)$, where experimental represents the low-, medium-, and high-intensity treatments and the control represents the single density noncourting treatment). A Dixon test for outliers was performed on the final ΔCt and $\Delta\Delta Ct$ estimates and resulting outliers were discarded ($\alpha = 0.05$; Sokal and Rohlf 1995). To detect differences between the treatments in the pre-mating episode, we employed a nonparametric Wilcoxon rank sum test at each time interval for each gene. To detect differences between courtship treatments, we employed a nonparametric Kruskal–Wallis test for each gene. In addition, we used a one-sample t-test to detect if any courtship treatments were significantly different from 1 (represents an equivalent $\Delta\Delta Ct$ between the courting treatment and the noncourting control group). No difference in control gene expression was detected between the treatments in either experiment (all $P > 0.25$). All analyses were performed in JMP8.

Results

During the premating episode, all three seminal fluid proteins in both treatments exhibited a sharp increase in gene expression ($2^{\Delta Ct}$) from eclosion to 24 h post-eclosion (Fig. 1). After 24 h, the level of gene expression began to vary according to the male treatment and the gene of interest. For Acp26Aa, males in the high-intensity treatment showed a small increase in expression at 24 h post-eclosion ($\chi^2_1 = 4.84$, $P = 0.0277$) compared with the low-intensity treatment. However, the high-intensity treatment exhibited relatively large decreases in expression both 48 and 72 h post eclosion ($\chi^2_1 = 4.43$, $P = 0.0353$ and $\chi^2_1 = 4.01$, $P = 0.0452$, respectively). There was a similar trend for Acp62f (t_{48} : $\chi^2_1 = 3.02$, $P = 0.0821$; t_{72}: $\chi^2_1 = 4.03$, $P = 0.0446$). Although expression levels for Acp70A appeared to be lower for the high-intensity males, this difference was marginally insignificant (t_{48} : $\chi^2_1 = 2.71$, $P = 0.0996$; t_{72}: $\chi^2_1 = 2.26$, $P = 0.1331$). At 72 h after eclosion, Acp26Aa exhibited a 36% reduction in production, while Acp62f exhibited a 35% reduction in production. Conservatively, Acp70 exhibited only an 18% reduction at the same

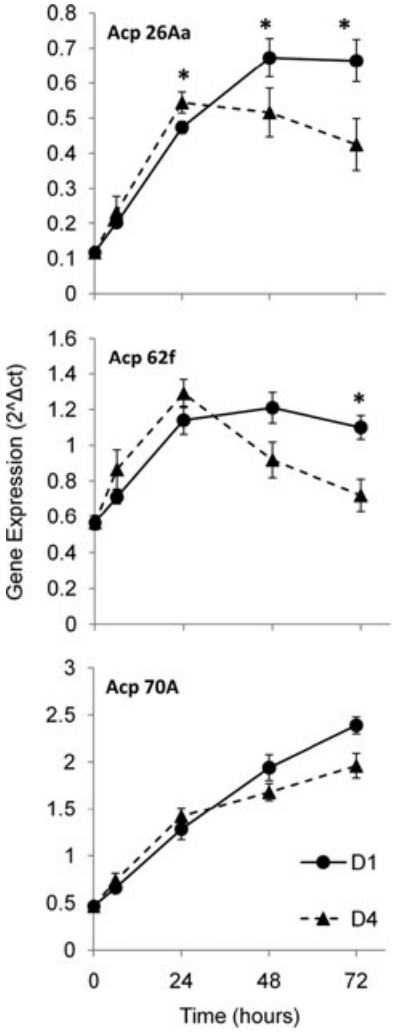

Figure 1. Expression of Sfp genes in the pre-mating episode. Males in the high competition-intensity treatment (triangles) exhibited a decline in gene expression 72 h after adult eclosion for Acp26Aa and Acp62f (Acp70A was marginally insignificant). Asterisk denotes a significant difference between the treatments for a given time point ($P < 0.05$).

time point (the difference between the treatments may actually be zero, considering that no statistical difference was detected for Acp70A). Thus, Acp26Aa and 62f saw at least a twofold greater reduction in production than Acp70A, suggesting that males alter Sfp ratios as well as Sfp production.

As with the Sfps, the spermatogenesis genes exhibited a steep increase in gene expression over the first 24 h post eclosion, which continued throughout the course of the experiment (Fig. 2). However, no difference in expression between the treatments was detected for any of the spermatogenesis genes (Fig. 2; all $P > 0.05$). Overall, the pre-mating data suggest that males in the high-intensity treatment produced similar amounts of spermatogenesis proteins, but fewer seminal fluid proteins, when compared with the low-intensity treatment.

During the courtship episode, rival male presence had no influence on Sfp (all $P > 0.19$) and spermatogenesis (all $P > 0.5$) gene regulation. Furthermore, no difference was detected between the courtship treatments and noncourting controls (one-sample *t*-test: all $P > 0.1$), suggesting that female presence also had no influence on gene regulation. Interestingly, Sfp gene expression exhibited nearly twice the coefficient of variation as did the spermatogenesis genes (CV = 29.6% ± 2.8% vs. 16.7% ± 1.7%, mean ± se, respectively; $\chi^2_1 = 9.79$, $P = 0.0018$). No difference in variation was between Sfp and spermatogenesis genes observed during the pre-mating episode (CV = 19.8% ± 2.1% vs. 24.4% ± 1.8%, mean ± se, respectively; $\chi^2_1 = 2.79$, $P = 0.0948$).

Discussion

In this study, we address the potential for males to modify the production of their ejaculate in accordance with perceived sperm competition intensity during the pre-mating and courtship phase of reproduction. In the pre-mating episode, all Sfps exhibited a sharp increase over the first 24 h, while spermatogenesis genes increased over 72 h. This pattern suggests that male fertility may be limited over the first several days of adulthood until ejaculate production and storage reaches its peak. In addition, high-intensity males tended to invest less in Acp26Aa and Acp62f, while investment in Acp70A and the spermatogenesis genes were consistent between the treatments (suggesting that our results were not a simple density effect). Interestingly, males also appear to modify Sfps ratios, although further study is needed to confirm this pattern. In the courtship episode, no difference between the treatments was detected for either spermatogenesis or seminal fluid proteins. Our data suggest that perceived sperm competition intensity had a significant influence on Sfp production in the pre-mating environment, but that males may have little ability to modify their ejaculate via protein production once courtship begins.

Recent work in *D. melanogaster* also suggests that extended exposure to rival males prior to mating influences ejaculate allocation, but in the form of increased ejaculate donation during mating (Bretman et al. 2009, 2010; Wigby et al. 2009). When combined with our current data, it appears that high intensity males produce fewer Sfps in the pre-mating environment, but donate more Sfps during mating. One explanation for this difference is that the decline in Sfp production is not a direct response to sperm competition, but represents a trade-off between Sfps and other costly traits associated with a high-density social environment, such as male fighting. If true, then high intensity males may incur a reproductive cost in the form of an increased refractory period between matings or an increased rate of Sfp depletion. Thus, future work should examine the long-term reproductive effects of a high intensity sperm competitive environment. It should

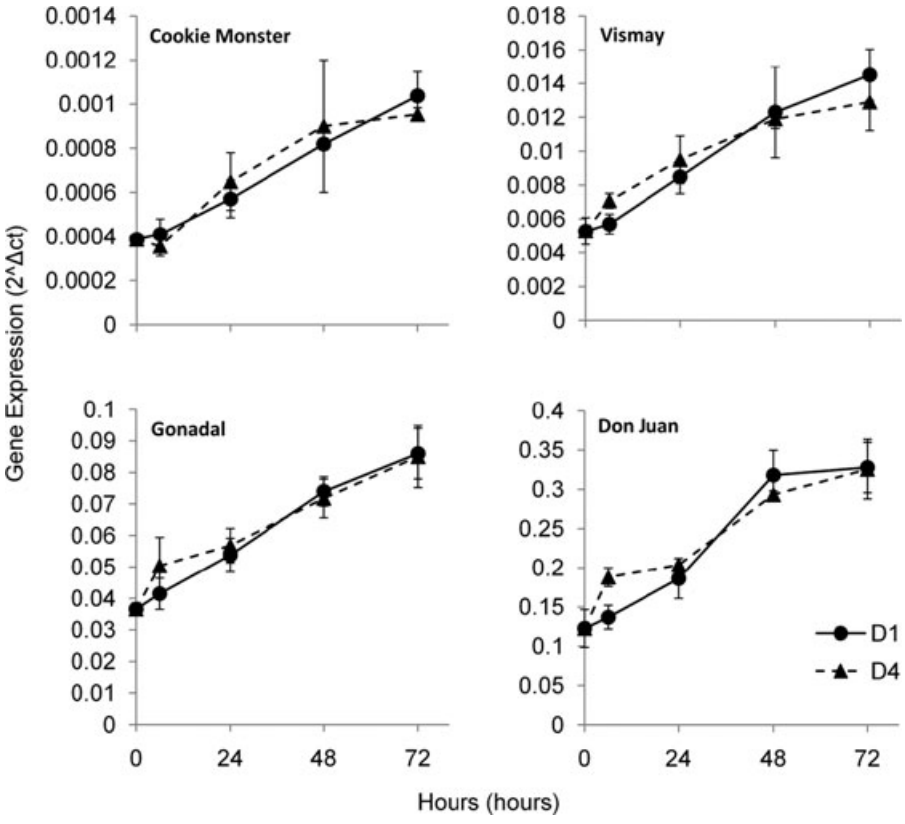

Figure 2. Expression of spermatogenesis genes in the pre-mating episode. No difference was detected between the high (triangles) and low (circles) competition-intensity treatments over the first 72 h post-adult eclosion for any of the genes associated with spermatogenesis. Asterisk denotes a significant difference between the treatments for a given time point ($P < 0.05$).

also be noted that Sfp mRNA levels may not accurately reflect male reproductive potential, as has been suggested by previous work in this system (McGraw et al. 2007). Therefore, future work should also examine male protein levels under varying degrees of competition in the pre-mating environment.

Interestingly, our pre-mating results appear to be consistent with Cameron et al. (2007), which is the only sperm competition model that addresses strategic allocation of both sperm and non-sperm components of the ejaculate. If Sfps generally influence female fecundity, then this model predicts that males disfavored in competition should invest less in Sfps. The underlying reason is that fecundity enhancing proteins can be viewed as communal products that potentially benefit subsequent sperm donation from rival males (Hodgson and Hosken 2006), whereas sperm only benefit the donor. Accordingly, our data indicate high intensity males maintain sperm production while reducing the production of certain seminal proteins. However, the comparison of any empirical data with general sperm competitive models should be approached with caution, as theoretical predictions are likely to be modified by system-specific aspects of reproduction.

As stated previously, our results indicate that males do not modify their ejaculate production during courtship when rivals are present, or even in response to female presence. Although this is surprising, it is consistent with a recent study examining the transcriptome response to social interaction in *D. melanogaster* (Carney 2007). In this study, males were singly paired with females and allowed to court for five minutes. Although 43 loci were identified with altered gene expression via a gene chip array, none of these were associated with Sfps or spermatogenesis after validating the results with real-time qPCR. These data further support the idea that males make significant ejaculate allocation decisions prior to the courtship and mating episodes.

In summary, our data suggest that males strategically produce seminal fluid proteins in response to perceived sperm competition intensity, and that these results appear to conform roughly to theoretical predictions. Specifically, our results indicate that (1) competition in the pre-mating environment significantly influences Sfp production, (2) Sfp production is significantly decreased under high competition intensity, however, sperm production remained unchanged, (3) males modify Sfp ratios in response to competition intensity, and (4) males do not modify ejaculate production during courtship in response to either rival male or female presence. The patterns presented here may be a general trend in promiscuous animal systems with internal fertilization. Thus, future work should aim at understanding the relative contributions of the different reproductive episodes on sperm and Sfp

production (and donation) across multiple animal systems. Furthermore, future work should examine the potential modification of Sfp ratios during Sfp production and donation.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Expression of Sfp genes in the courtship episode.
Figure S2. Expression of spermatogenesis genes in the courtship episode.

Supporting Information may be found in the online version of this article.