

Selection on the *Drosophila* seminal fluid protein Acp62F

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Introduction

In a broad range of taxa including vertebrates, invertebrates, fungi, and plants, some of the most rapidly evolving proteins encoded in the genome are those contributing to reproductive success (for reviews, see Clark et al. 2006; Chapman 2008; Wong 2011). In *Drosophila*, for example, seminal fluid proteins (SFPs) that are transferred from males to females during copulation diverge quickly at the sequence level, and the proportion of SFP-encoding genes subject to positive selection is unusually high compared with the rest of the genome (e.g., Civetta and Singh 1995; Haerty et al. 2007). Moreover, the complement of SFPs produced by different species can vary drastically, with apparently high rates of gene loss and recruitment of new genes (e.g., Holloway and Begun 2004; Mueller et al. 2005; Wagstaff and Begun 2005a, 2007; Haerty et al. 2007; Findlay et al. 2008; Kelleher et al. 2009).

Several hypotheses have been proposed to explain the rapid evolution of reproductive proteins. In internally fertilizing animals, a leading proposal is that postcopulatory

Abstract

Sperm competition and sexual conflict are thought to underlie the rapid evolution of reproductive proteins in many taxa. While comparative data are generally consistent with these hypotheses, few manipulative tests have been conducted and those that have provided contradictory results in some cases. Here, we use both comparative and experimental techniques to investigate the evolution of the *Drosophila melanogaster* seminal fluid protein Acp62F, a protease inhibitor for which extensive functional tests have yielded ambiguous results. Using between-species sequence comparisons, we show that Acp62F has been subject to recurrent positive selection. In addition, we experimentally evolved populations polymorphic for an *Acp62F* null allele over eight generations, manipulating the opportunities for natural and sexual selection. We found that the *Acp62F* null allele increased in frequency in the presence of natural selection, with no effect of sexual selection.

sexual selection is responsible, for example, via sperm competition or sexual conflict. Sperm competition arises when sperm from multiple males are present at the same time in the reproductive tract of a female. If an SFP variant leads to an increased paternity share, then that variant should be favored by sexual selection. Consistent with an effect of sperm competition on SFP evolution, several rapidly evolving *Drosophila melanogaster* SFP genes have known effects on sperm storage and/or sperm competition (Acp36DE: Neubaum and Wolfner 1999; Chapman et al. 2000; Acp29AB: Wong et al. 2008; CG9997: Ram and Wolfner 2007).

Similarly, a number of *Drosophila* SFPs are known to have effects on processes potentially involved in interlocus sexual conflict. Interlocus sexual conflict arises when the optimal outcome of an interaction between the sexes is different for males and females. For example, females of some species may gain by mating with multiple males (Simmons 2005) owing to sperm depletion (e.g., Thornhill and Alcock 1983), sperm quality (e.g., Keller and Reeve 1995), and other indirect benefits, and/or from direct benefits such as

nuptial gifts (e.g., Simmons et al. 1999). By contrast, it is typically in a male's interest that his partner does not mate with other males. As such, the optimal remating rate may be higher for females than it is for males (e.g., Gavrillets and Hayashi 2006). In *D. melanogaster*, the "sex-peptide (SP) network" of proteins modulates remating rate, as well as several other postmating responses (Chapman et al. 2003; Liu and Kubli 2003; Ram and Wolfner 2009; LaFlamme et al. 2012), and at least one member of the network has been subject to positive selection (CG9997; Wong et al. 2012). The SP network may in fact be involved in sexual conflict in several different ways; in addition to its effects on female remating, a functional SP network is necessary for the mating-induced reduction in female life span observed in this species (Wigby and Chapman 2005).

Consistent with the hypothesis that sexual selection is a primary cause of rapid reproductive protein evolution, comparative studies indicate a correlation between mating system and the rates of evolution of some reproductive proteins, including SFPs. That is, for some individual proteins, rates of evolution are higher in polyandrous lineages than in monandrous lineages, where postcopulatory sexual selection is less likely to occur (e.g., Ramm et al. 2008; Finn and Civetta 2010; Prothmann et al. 2012). Moreover, average rates of reproductive protein evolution are higher in polyandrous lineages in comparison with monandrous lineages in primates and in *Drosophila* (Wagstaff and Begun 2005b, 2007; Kelleher et al. 2007; Almeida and Desalle 2009), again suggesting that sexual conflict is important in the evolution of reproductive proteins. Additional sequence-based evidence for an effect of sexual selection on rates of reproductive protein evolution comes from Clark et al. (2009), who showed strong linkage disequilibrium between the abalone sperm protein lysin and its egg-receptor vitelline envelope receptor for lysin, as predicted under models of sexual selection.

In this study, we investigated the evolution of the *D. melanogaster* SFP *Acp62F*. *Acp62F* has been extensively studied using genetic and biochemical methods, but its function nevertheless remains unclear. The *Acp62F* protein is a protease inhibitor (Lung et al. 2002), a biochemical characteristic that it shares with numerous other SFPs (Mueller et al. 2004; Laflamme and Wolfner 2012). Overexpression of *Acp62F* in larvae or adults is toxic (Lung et al. 2002; Mueller et al. 2007), and a QTL containing the *Acp62F* locus is associated with postmating female mortality in interspecific introgression lines (Civetta et al. 2005), suggesting that this protein may be involved in seminal fluid toxicity. If so, then one would expect this protein to have other fitness-enhancing effects that outweigh its costs to females (unless toxicity is itself beneficial to males – see Johnstone and Keller 2000). Such a benefit has not been identified; indeed, Mueller et al. (2008) found that *Acp62F* knockout males performed *better* in sperm competition than did wild-type (wt) males.

Mueller et al. (2008) also showed that *Acp62F* is required for normal proteolytic cleavage of the egg-laying hormone Ovulin, but the functional consequences of this phenotype are unclear as mates of *Acp62F* knockout males did not show any obvious defect in egg laying.

Here, we use computational and manipulative approaches to investigate the nature of selection acting on *Acp62F*. Using comparative sequence analyses, we test for a signature of positive selection on *Acp62F*. Furthermore, in a two-way factorial evolution experiment, we independently manipulate the opportunities for selection arising from variation in sexual and nonsexual fitness in replicate experimental populations polymorphic for *Acp62F* wt and null alleles, and then determine the consequences of this manipulation by tracking changes across generations in allele frequencies. This approach allows the net effects of *Acp62F* on sexual and nonsexual fitness to be integrated by the evolutionary process itself.

Materials and Methods

Inference of selection from comparative sequence data

We obtained nucleotide sequences of *Acp62F* from *D. melanogaster*, and orthologous sequences from *Drosophila simulans*, *Drosophila sechellia*, *Drosophila yakuba*, and *Drosophila erecta* from FlyBase (FlyBase IDs: FBgn0020509, FBgn0043400, FBgn0069552, FBgn0107140, FBgn0237654). Translated sequences were aligned using Muscle (Edgar 2004) and back translated to nucleotide sequences using T-COFFEE (Notredame et al. 2000). The resulting multiple sequence alignment is included in Appendix 1.

We used three methods to infer recurrent positive selection on *Acp62F*. The first method implements the "sites" models in codeml, part of the PAML package (Yang 2007). Findlay et al. (2008) conducted the same analysis; we replicate it here to confirm their results. The sites models allow variation in ω (dN/dS) between different codons within a gene, but assume that all lineages experience the same distribution of ω . Two null models were used, M7 and M8A (Yang et al. 2000), each of which restricts ω to be less than or equal to one, thus disallowing positive selection. M7 assumes a beta-distribution for ω and M8A assumes a beta-distribution as well as an extra class of sites in which $\omega = 1$. The alternative model M8 assumes a beta-distribution for ω (restricted to be ≤ 1), but adds a class of sites with $\omega \geq 1$. M8 can be compared with either null model via a likelihood ratio test (with 2 and 1 df for M7 and M8A, respectively), and a significant rejection of the null model is evidence in favor of positive selection on a subset of codons.

We additionally used the random-effects likelihood (REL) and fixed-effects likelihood (FEL) methods of Kosakovsky

Pond and Frost (2005) as more robust tests for positive selection on *Acp62F*. REL and FEL analyses were performed on the hypothesis testing using phylogenies datamonkey server (Pond and Frost 2005). These methods allow variation in both dN and dS , whereas the models implemented in PAML assume a single value of dS . REL assumes predefined distributions for dN and dS , and after initial inference of parameter values uses an empirical Bayes approach to infer selection on each site. As such, like PAML, the REL analysis assigns to each codon a posterior probability that it is under positive selection, with higher posterior probabilities indicating greater confidence that selection operates on the given codon. FEL, in contrast, directly estimates dN and dS at each site. Simulation results suggest that REL may be subject to higher false-positive rates for alignments with few species, such as the five-species alignment used here, whereas FEL does not seem to suffer from this problem (Kosakovsky Pond and Frost 2005). For each codon, FEL estimates the probability of obtaining the observed dN and dS values under a neutral model (i.e., a P -value under neutrality), with lower P -values indicating rejection of neutrality in favor of positive selection.

Experimental evolution of populations polymorphic for an *Acp62F* null allele

An inbred stock of *D. melanogaster* homozygous for an *Acp62F* null allele ($\Delta Acp62F$) on a w^{1118} background, as well as a genotype-matched w^{1118} *wt* allele, was kindly provided by N. Buehner and M. F. Wolfner. The $\Delta Acp62F$ allele was previously described by Mueller et al. (2008), and was constructed using the precise gene-targeting method of Rong and Golic (2000). We confirmed the *Acp62F* genotypes of both the *wt* and $\Delta Acp62F$ lines prior to the beginning of the experiment. All populations were maintained on a 12-h light:dark cycle on cornmeal food at 25°C.

Twelve replicate populations were maintained throughout the course of the experiment. Each population was started with the *Acp62F* null and *wt* alleles each at a frequency of 0.5, with genotypes at Hardy–Weinberg equilibrium. Three populations were assigned to each of the four possible treatments in a factorial design following Rundle et al. (2006): natural and sexual selection both present (NS), natural selection present and sexual selection greatly reduced (Nx), natural selection greatly reduced and sexual selection present (xS), or natural and sexual selection both reduced (xx). In populations in which sexual selection was reduced, a single sexually mature virgin female and a single mature male (both aged 3–4 days) were placed in a vial together for 2–3 days to mate. In populations in which sexual selection was present, a single virgin female was housed with 3–5 males for the same period of time for mating, thus allowing both pre- and postcopulato-

ry sexual selection. After mating, males were discarded and 50 females per population were individually placed in separate vials to lay eggs for 2–3 days, after which the females were frozen at -80°C . For populations in which natural selection was reduced, each female contributed one female offspring and one (sexual selection reduced) or four (sexual selection present) male offspring to the next generation. For natural selection present treatments, all offspring produced by all females were pooled and 50 females and 50 (or 200) males were randomly selected for the next generation, such that each female contributed to the next generation in proportion to her productivity. The natural selection treatment thus incorporated virtually all nonsexual components of selection, including female fecundity, male fecundity, and egg-adult survival of offspring.

Genotyping

DNA was extracted from individual frozen females at generations 6 and 8 following the method of Gloor et al. (1993). Single flies were ground in 50 μL of squish buffer (10 mmol/L Tris-Cl pH 8.2, 1 mmol/L EDTA, 25 mmol/L NaCl, and 200 $\mu\text{g}/\text{mL}$ proteinase K) and incubated for 1 h at 37°C. Proteinase K was then inactivated at 95°C for 5 min, samples were centrifuged, and the supernatant was used as a template for subsequent polymerase chain reaction (PCR) reactions.

By PCR 12–15 individuals were genotyped from each population at generation 6 and at generation 8. Two primer pairs were used: primer pair 1 (*Acp62F*-screen1 and *Acp62F*-screen2 from Mueller et al. 2008) consisted of primers flanking the *Acp62F* locus, and in principle should generate a long (1635 bp) PCR product from the *wt* allele, and a short (825 bp) allele from the $\Delta Acp62F$ allele. However, we found that amplification of the long *wt* allele was inconsistent, particularly in heterozygotes. Thus, we additionally used primer pair 2 (*Acp62F*-4: ACTGGGCAGCAGGTGGAATG; *Acp62F*-5: CGAACTTT AAGTGCTTTAGCAG), consisting of one primer within the *Acp62F* gene, and one in the downstream flank. This primer pair consistently amplified a 275 bp fragment from the *wt* allele, and produced no product from the knockout allele. All samples were genotyped twice with each primer pair to ensure reproducibility, and samples producing no PCR product with either primer pair were discarded. Remaining individuals were scored as homozygous for either the null or *wt* *Acp62F* allele, or as heterozygotes. Raw genotype data are provided in Appendix 2.

Statistical analyses

Counts of the *Acp62F* *wt* and null alleles for each population sample were calculated from the genotype data.

Effects of natural selection, sexual selection, and their interaction on allele counts were assessed via binomial regression, using a call to the `glm()` function in R (R Core Development Team 2011).

Results and Discussion

Comparative evidence for positive selection on *Acp62F*

Population-genetic and comparative sequence analyses have documented positive selection on many genes encoding *Drosophila* SFPs (reviewed in Civetta 2003; Clark et al. 2006; Chapman 2008; Wong and Wolfner 2012). In keeping with this broad trend, we find evidence of positive selection on *Acp62F* (Tables 1 and 2) using sequence data from five *Drosophilid* species. Using the PAML package (Yang 2007), we find that the data fit a model (M8) that allows positive selection on a subset of codons significantly better than either of the two null models that do not allow positive selection. Under M8, 27% of codons fall into the positively selected class, with $\omega = 2.92$. Analysis of the same dataset using the REL method of Kosakovsky Pond and Frost (2005), which is more robust to synonymous site rate variation, also provides evidence for positive selection (Table 2), with five codons inferred to have $\omega > 1$ with a posterior probability

Table 1. Statistical evidence for positive selection on *Acp62F* from PAML.

Model	Log likelihood	$-2\Delta\ln L$ versus M8	<i>P</i> -value	Proportion selected sites	ω
M8	−1044.84	NA	NA	0.27	2.92
M8A	−1051.78	13.88	1.9×10^{-4}	NA	NA
M7	−1052.20	14.72	6.4×10^{-4}	NA	NA

Table 2. Sites under positive selection under the codon models M8, FEL, and REL.

Site	M8 posterior probability	REL posterior probability	FEL <i>P</i> -value
40	0.93	0.96	>0.5
42	0.99	0.97	0.13
45	0.90	0.99	0.08
99	0.94	0.97	0.30
100	0.91	<0.5	>0.5
103	0.99	0.62	>0.5
113	0.95	0.96	0.23
115	0.99	0.78	>0.5

For M8 and REL, posterior probabilities that a site is under positive selection are given, with values >0.9 highlighted in bold. For FEL, *P*-values give the probability that the site is *not* under positive selection, that is, the *P*-value under neutrality.

of >0.9. Analysis using the FEL method (Kosakovsky Pond and Frost 2005) provides less convincing evidence of selection, with only one site showing marginal evidence for an elevated *dN/dS* ratio (site 45; *P* = 0.08; Table 2). This discrepancy may suggest that the limited number of species used here produces false positives with PAML and with the REL method; alternatively, there may be insufficient power to robustly infer selection using FEL.

Acp62F is a known protease inhibitor (Lung et al. 2002), and knockout of *Acp62F* in males has been shown to slow the proteolysis of at least one other SFP in mated females (Mueller et al. 2008). Protease inhibition is thought to occur via mimicry of the target protease's normal substrate, with the inhibitor's reactive center loop (RCL) cleaved at the "P1" location. *Acp62F*'s predicted P1 residue is at position 61 (Lys⁶¹) of the *D. melanogaster* protein, with the predicted RCL consisting of residues within approximately five sites on either side of P1. None of the sites for which positive selection is inferred lie within the RCL (Table 2), suggesting that direct interactions with the target protease(s) do not underlie selection on *Acp62F*. Rather, we hypothesize that the selected sites are involved in nonreactive site interactions with target proteases, or that changes at these residues alter the shape and/or reaction kinetics of the RCL.

Findlay et al. (2008) also documented evidence of positive selection on *Acp62F* as part of a large-scale survey of the molecular evolution of *Drosophila* seminal proteins identified via mass spectrometry, using sequence data from the same set of species used here. Our results extend Findlay et al.'s inference of positive selection using more sophisticated statistical models (the REL and FEL models of Kosakovsky Pond and Frost 2005), and further the analysis of the causes of selection on this protein.

Experimental response to natural, but not sexual, selection

While statistical analyses of sequence data indicate a history of positive selection on *Acp62F*, they provide little insight into the specific processes underlying selection. Several studies have examined the effects of *Acp62F* on phenotypes known to be mediated by *Drosophila* seminal proteins, but have failed to find a clear benefit to producing *Acp62F*. In particular, knockout mutants have *improved* sperm competitive ability (Mueller et al. 2008), and induced overexpression of *Acp62F* is toxic in pre-adult and adult flies (Lung et al. 2002). We therefore used an experimental strategy to infer the effects of *Acp62F* on net sexual and nonsexual fitness. We manipulated the opportunities for natural and sexual selection in a two-way factorial design, tracking the frequency of the *Acp62F* wt and null alleles. We found a strong effect of natural

selection, with the null allele occurring at higher frequency in the presence than the absence of natural selection at both generation 6 and generation 8 (Fig. 1). For both treatments in which natural selection was unimpeded (Nx and NS), the frequency of the wt allele decreased over time relative to its starting frequency of 0.5, regardless of the presence or absence of sexual selection (NS and Nx, respectively, in Fig. 1). By contrast, for populations wherein natural selection was greatly reduced, the frequency of the wt allele was slightly elevated at generation 8 relative to its initial value. In a binomial regression, natural selection present/absent was a significant predictor of allele counts at both generation 6 and generation 8, with neither sexual selection present/absent nor their interaction being significant (Table 3).

Our failure to find an effect of *Acp62F* genotype on sexual fitness contrasts with results of a previous study examining the roles of *Acp62F* in various components of

sexual and nonsexual fitness. Mueller et al. (2008) found that sperm from *Acp62F* null males were better able to resist displacement by a second male's sperm (i.e., P1 was higher for null males). Thus, it might have been expected that the *Acp62F* null allele would have increased in frequency in the sexual selection present treatments in our study, but this was not observed. Differences in experimental design between our study and that of Mueller et al. (2008) may help to explain these apparently contradictory results. Whereas Mueller et al. (2008) assayed a number of fitness components in isolation, the experimental evolution protocol used here integrates over all components of fitness, including female productivity, male mating success, and male sperm competitive ability. Thus, any sperm competitive advantage held by *Acp62F* null males in our study may have been offset by fitness deficits in other components of sexual fitness. Other authors have also reported differences in the fitness effects of individual mutations in assays of single-fitness components as compared with integrated measures of fitness (e.g., Arbuthnott and Rundle 2012).

Our finding that the *Acp62F* null allele is favored by natural selection, as well as a previous study finding a benefit for the null allele in sperm competition (Mueller et al. 2008), raises the question as to why a functional *Acp62F* gene persists in natural populations. There are at least two possible explanations. First, the laboratory environment may impose or relieve constraints present in natural populations, or otherwise alter conditions such that the effects of *Acp62F* genotype on laboratory fitness may not fully reflect the natural setting. For example, while remating was possible during our experiment, rates may have been lower than those in natural populations due to the truncated adult life span. Second, because we used an isogenic, rather than an outbred, background (as did Mueller et al. 2008), the results obtained here may not be fully representative of *Acp62F*'s average fitness effect in an outbred population.

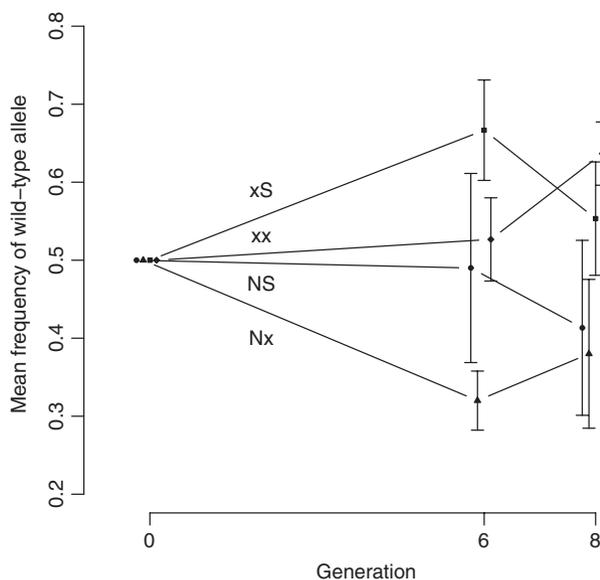


Figure 1. Changes in *Acp62F* wt allele frequency over time. Three populations were maintained under each combination of natural (N) and sexual (S) selection. Points represent the mean of the three populations, \pm 1 standard error.

Table 3. Effects of natural and sexual selection on *Acp62F* allele counts.

Factor	Generation 6			Generation 8		
	Estimate	SE	P-value	Estimate	SE	P-value
Natural selection	-0.868	0.346	0.012	-1.035	0.322	0.001
Sexual selection	0.582	0.344	0.091	-0.285	0.321	0.374
N \times S	0.117	0.489	0.811	0.401	0.450	0.373

Sexual selection neither reinforces nor inhibits natural selection on *Acp62F*

Our data are also relevant to the effects of sexual selection on nonsexual fitness, a topic of recent interest for which empirical data are mixed (Whitlock and Agrawal 2009). On the one hand, there are several ways by which sexual selection may reduce population mean fitness, including via the evolution of costly sexual displays and preferences for them, by generating sex-specific selection that leads to sexual conflict, and by reducing effective population size and thus increasing drift load. Alternatively, sexual selection may promote adaptation and the purging of deleterious alleles by inducing positive assortative mating for

fitness, via a good genes process (i.e., the evolution of mate preferences for individuals of high breeding value for fitness), or more generally if reproductive success is condition-dependent (Whitlock and Agrawal 2009). Manipulating the opportunity for sexual selection and then tracking the frequency of individual alleles during experimental evolution provides a powerful and straightforward approach to comparing such costs and benefits. To our knowledge this approach has been applied in only two cases to date, both in *D. melanogaster*. One experiment used an alcohol dehydrogenase (*Adh*) null allele and provided results consistent with a benefit of sexual selection (Hollis et al. 2009); the other used six independent recessive mutations with visible phenotypic effects, and results suggested no benefit, and in some cases a cost, of sexual selection (Arbuthnott and Rundle 2012). Results of the current experiment using *Acp62F* provide another example of the latter, with no evidence that sexual selection favored the null allele with the highest nonsexual fitness. It is important to keep in mind, however, that *Acp62F* was not randomly chosen with respect to the potential alignment of natural and sexual selection, given an a priori expectation of ongoing sexual conflict (although no evidence suggesting such conflict was found).

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Conflict of Interest

None declared.

References

- Almeida, F. C., and R. Desalle. 2009. Orthology, function and evolution of accessory gland proteins in the *Drosophila* repleta group. *Genetics* 181:235–245.
- Arbuthnott, D., and H. D. Rundle. 2012. Sexual selection is ineffectual or inhibits the purging of deleterious mutations in *Drosophila melanogaster*. *Evolution* 66:2127–2137.
- Chapman, T. 2008. The soup in my fly: evolution, form and function of seminal fluid proteins. *PLoS Biol.* 6:e179.
- Chapman, T., D. M. Neubaum, M. F. Wolfner, and L. Partridge. 2000. The role of male accessory gland protein *Acp36DE* in sperm competition in *Drosophila melanogaster*. *Proc. Biol. Sci.* 267:1097–1105.
- Chapman, T., J. Bangham, G. Vinti, B. Seifried, O. Lung, M. F. Wolfner, et al. 2003. The sex peptide of *Drosophila melanogaster*: female post-mating responses analyzed by using RNA interference. *Proc. Natl Acad. Sci. USA* 100:9923–9928.
- Civetta, A. 2003. Shall we dance or shall we fight? Using DNA sequence data to untangle controversies surrounding sexual selection. *Genome* 46:925–929.
- Civetta, A., and R. Singh. 1995. High divergence of reproductive tract proteins and their association with postzygotic reproductive isolation in *Drosophila melanogaster* and *Drosophila virilis* group species. *J. Mol. Evol.* 41:1085–1095.
- Civetta, A., K. L. Montooth, and M. Mendelson. 2005. Quantitative trait loci and interaction effects responsible for variation in female postmating mortality in *Drosophila simulans* and *D. sechellia* introgression lines. *Heredity* 94:94–100.
- Clark, N. L., J. E. Aagaard, and W. J. Swanson. 2006. Evolution of reproductive proteins from animals and plants. *Reproduction* (Cambridge, England) 131:11–22.
- Clark, N. L., J. Gasper, M. Sekino, S. A. Springer, C. F. Aquadro, and W. J. Swanson. 2009. Coevolution of interacting fertilization proteins. *PLoS Genet.* 5:e1000570.
- Edgar, R. C. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 5:113.
- Findlay, G. D., X. Yi, M. J. Maccoss, and W. J. Swanson. 2008. Proteomics reveals novel *Drosophila* seminal fluid proteins transferred at mating. *PLoS Biol.* 6:e178.
- Finn, S., and A. Civetta. 2010. Sexual selection and the molecular evolution of ADAM proteins. *J. Mol. Evol.* 71:231–240.
- Gavrillets, S., and T. I. Hayashi. 2006. The dynamics of two- and three-way sexual conflicts over mating. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 361:345–354.
- Gloor, G. B., C. R. Preston, D. M. Johnson-Schlitz, N. A. Nassif, R. W. Phillis, W. K. Benz, et al. 1993. Type I repressors of P element mobility. *Genetics* 135:81–95.
- Haerty, W., S. Jagadeeshan, R. J. Kulathinal, A. Wong, K. Ravi Ram, L. K. Sirot, et al. 2007. Evolution in the fast lane: rapidly evolving sex-related genes in *Drosophila*. *Genetics* 177:1321–1335.
- Hollis, B., J. L. Fierst, and D. Houle. 2009. Sexual selection accelerates the elimination of a deleterious mutant in *Drosophila melanogaster*. *Evolution* 63:324–333.
- Holloway, A. K., and D. J. Begun. 2004. Molecular evolution and population genetics of duplicated accessory gland protein genes in *Drosophila*. *Mol. Biol. Evol.* 21:1625–1628.
- Johnstone, R. A., and L. Keller. 2000. How males can gain by harming their mates: sexual conflict, seminal toxins, and the cost of mating. *Am. Nat.* 156:368–377.
- Kelleher, E. S., W. J. Swanson, and T. a. Markow. 2007. Gene duplication and adaptive evolution of digestive proteases in

- Drosophila arizonae* female reproductive tracts. *PLoS Genet.* 3:e148.
- Kelleher, E. S., T. D. Watts, B. A. LaFlamme, P. A. Haynes, and T. A. Markow. 2009. Proteomic analysis of *Drosophila mojavensis* male accessory glands suggests novel classes of seminal fluid proteins. *Insect Biochem. Mol. Biol.* 39: 366–371.
- Keller, L., and H. K. Reeve. 1995. Why do females mate with multiple males - The sexually selected sperm hypothesis. *Adv. Study Behav.* 24:291–315.
- Kosakovsky Pond, S. L., and S. D. Frost. 2005. Not so different after all: a comparison of methods for detecting amino acid sites under selection. *Mol. Biol. Evol.* 22:1208–1222.
- Laflamme, B. A., and M. F. Wolfner. 2012. Identification and function of proteolysis regulators in seminal fluid. *Mol. Reprod. Dev.* 80:80–101.
- LaFlamme, B., K. Ravi Ram, and M. Wolfner. 2012. The *Drosophila melanogaster* seminal fluid protease “seminase” regulates proteolytic and post-mating reproductive processes. *PLoS Genet.* 8:e1002435.
- Liu, H., and E. Kubli. 2003. Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* 100:9929–9933.
- Lung, O., U. Tram, C. M. Finnerty, and M. a. Eipper-Mains, J. M. Kalb, and M. F. Wolfner.. 2002. The *Drosophila melanogaster* seminal fluid protein Acp62F is a protease inhibitor that is toxic upon ectopic expression. *Genetics* 160:211–224.
- Mueller, J. L., D. R. Ripoll, C. F. Aquadro, and M. F. Wolfner. 2004. Comparative structural modeling and inference of conserved protein classes in *Drosophila* seminal fluid. *Proc. Natl Acad. Sci. USA* 101:13542–13547.
- Mueller, J. L., K. Ravi Ram, L. A. McGraw, M. C. Bloch Qazi, E. D. Siggia, A. G. Clark, et al. 2005. Cross-species comparison of *Drosophila* male accessory gland protein genes. *Genetics* 171:131–143.
- Mueller, J. L., J. L. Page, and M. F. Wolfner. 2007. An ectopic expression screen reveals the protective and toxic effects of *Drosophila* seminal fluid proteins. *Genetics* 175:777–783.
- Mueller, J. L., J. R. Linklater, K. Ravi Ram, T. Chapman, and M. F. Wolfner. 2008. Targeted gene deletion and phenotypic analysis of the *Drosophila melanogaster* seminal fluid protease inhibitor Acp62F. *Genetics* 178:1605–1614.
- Neubaum, D. M., and M. F. Wolfner. 1999. Mated *Drosophila melanogaster* females require a seminal fluid protein, Acp36DE, to store sperm efficiently. *Genetics* 153:845–857.
- Notredame, C., D. G. Higgins, and J. Heringa. 2000. T-Coffee: a novel method for fast and accurate multiple sequence alignment. *J. Mol. Biol.* 302:205–217.
- Pond, S. L., and S. D. Frost. 2005. Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. *Bioinformatics* 21:2531–2533.
- Prothmann, A., I. Laube, J. Dietz, C. Roos, K. Mengel, H. Zischler, et al. 2012. Sexual size dimorphism predicts rates of sequence evolution of SPERM Adhesion Molecule 1 (SPAM1, also PH-20) in monkeys, but not in hominoids (apes including humans). *Mol. Phylogenet. Evol.* 63:52–63.
- Ram, K. R., and M. F. Wolfner. 2007. Sustained post-mating response in *Drosophila melanogaster* requires multiple seminal fluid proteins. *PLoS Genet.* 3:e238.
- Ram, K. R., and M. F. Wolfner. 2009. A network of interactions among seminal proteins underlies the long-term postmating response in *Drosophila*. *Proc. Natl Acad. Sci. USA* 106:15384–15389.
- Ramm, S. A., P. L. Oliver, C. P. Ponting, P. Stockley, and R. D. Emes. 2008. Sexual selection and the adaptive evolution of mammalian ejaculate proteins. *Mol. Biol. Evol.* 25:207–219.
- Rong, Y. S., and K. G. Golic. 2000. Gene targeting by homologous recombination in *Drosophila*. *Science (New York, N.Y.)* 288:2013–2018.
- Rundle, H. D., S. F. Chenoweth, and M. W. Blows. 2006. The roles of natural and sexual selection during adaptation to a novel environment. *Evolution* 60:2218–2225.
- Simmons, L. W. 2005. The evolution of polyandry: sperm Competition, Sperm Selection, and Offspring Viability. *Annu. Rev. Ecol. Evol. Syst.* 36:125–146.
- Simmons, L. W., L. Beesley, P. Lindhjem, D. Newbound, J. Norris, and A. Wayne. 1999. Nuptial feeding by male bushcrickets: an indicator of male quality? *Behav. Ecol.* 10:263–269.
- Team, R. C. D. 2011. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.
- Thornhill, R., and J. Alcock. 1983. The evolution of insect mating systems. Harvard University Press, Cambridge.
- Wagstaff, B. J., and D. J. Begun. 2005a. Comparative genomics of accessory gland protein genes in *Drosophila melanogaster* and *D. pseudoobscura*. *Mol. Biol. Evol.* 22:818–832.
- Wagstaff, B. J., and D. J. Begun. 2005b. Molecular population genetics of accessory gland protein genes and testis-expressed genes in *Drosophila mojavensis* and *D. arizonae*. *Genetics* 171:1083–1101.
- Wagstaff, B. J., and D. J. Begun. 2007. Adaptive evolution of recently duplicated accessory gland protein genes in desert *Drosophila*. *Genetics* 177:1023–1030.
- Whitlock, M. C., and A. F. Agrawal. 2009. Purging the genome with sexual selection: reducing mutation load through selection on males. *Evolution* 63:569–582.
- Wigby, S., and T. Chapman. 2005. Sex peptide causes mating costs in female *Drosophila melanogaster*. *Curr. Biol.* 15: 316–321.
- Wong, A. 2011. The molecular evolution of animal reproductive tract proteins: what have we learned from mating-system comparisons? *Int. J. Evol. Biol.* 2011:1–9.
- Wong, A., and M. F. Wolfner. 2012. Evolution of *Drosophila* seminal proteins and their networks. Pp. 144–152 in R. S. Singh, J. Xu and R. J. Kulathinal, eds. Rapidly evolving

genes and genetics systems. Oxford University Press, Oxford.

Wong, A., S. N. Albright, J. D. Giebel, K. R. Ram, S. Ji, A. C. Fiumera, et al. 2008. A role for Acp29AB, a predicted seminal fluid lectin, in female sperm storage in *Drosophila melanogaster*. *Genetics* 180:921–931.

Wong, A., M. Turchin, M. F. Wolfner, and C. F. Aquadro. 2012. Temporally variable selection on proteolysis-related

reproductive tract proteins in *Drosophila*. *Mol. Biol. Evol.* 29:229–238.

Yang, Z. 2007. PAML 4: phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* 24:1586–1591.

Yang, Z., R. Nielsen, N. Goldman, and A. M. Pedersen. 2000. Codon-substitution models for heterogeneous selection pressure at amino acid sites. *Genetics* 155: 431–449.

Appendix 1: FASTA alignment of Acp62F from five *Drosophila* species.

```
>Dmel
ATGACGGACATGTGGAGCTTGAAGATCTGTGCCGTGCTGGGCCCTTCTATTACTTTTCAAACCCATCGACTCCATGGGATG
GCAAGGACCTAAAGTTGACTGTACGGCCAACGGAACTCAGACGGAGTGTCCGTGTAGCATGTCTGAAACCTGCGAGTACT
CCGGCAATGGACCCTGCGTCAAGATGTGCGGAGCTCCTTGTGTGTGTAAGCCGGGATATGTTATCAATGAGAGGATTCCG
GCCTGTGTTCTGCGATCCGATTGCCAAAAGATGTTGTTTCGAAAGGAAGATATGCTACTGGGTGTATCGAACTTTAAGTG
CTTTAGCAGAAATTACAACCTGTTCA-----
-----
>Dyak
ATGTTGATAACGTGCACCTTGAAGATCTGTGCCCTGCTGGGCCCTCCTACTCCTTTTCAAACCCATCGAAAGTTGGGATTT
CGGC-----
TGTACCGTCAACGGAAGCCTAGCGTTCGTGTCCTTCAGCATGTCTGAAACCTGCGAGTACTCTGGTATAGGACCCTGCGT
AGATATGTGCGGCGGTCTTGTGTGTGTAAGCCGGGATATGTAATCAATGAGAGGATTCGGCTTGTGTTCTGCGATCAG
ATTGCCCCAAAGATGTGATTTCGTAAGGAACAAATGACAGAAGGTCATTCAAATTTTAAATGCTTTAGCAGAAATTTAAAG
TGTGTTGGG-----GAATTTTTTAAA-----ACAAGAAG-----
>Dsim
ATGACGGACATGTGGAGCTTGAAGATCTGTGCCGTGGCTGGGCCCTCCTATTACTTTTCAAACCCATCGACTCCACGGGCTT
GGAAGGACCTAATGTGACTGTACGGCCAACGGAACTCGGGCGGTGTGTCCTGTAGCATGTCTGAAACCTGCGCGTACT
CCGGCGATGGACCCTGCGTCAAGATGTGCGGAGCCCCGTGTTGTGTGTAAGCCGGGATATGTTATCAATGAAAGAATCCCT
GCCTGTGTCCTGCGATCCGATTGCCAAAAGATGTCGTTTCGAAAGGAAGATATGCTACTAGGTGTAACGAACTTTAAGTG
CTTTAGCAGAAATTACGTGTGTAAGAAT-----TTA-----
-----
>Dsec
ATGACGGACATAAGGAGCTGGAAAATCTGTGCCCTGGTGGGCCCTCCTATTACTTTTCAAACCCATCGACTCCACGGGCTT
GGAAGGACGTAATGTTGACTGTACGGCCAACGGAACTCGGGCGGTGTGTCCTGTAGCATGTCTGAAACCTGCGAGTACT
CCGGCGATGGACCCTGCGTCAAGGTGTGCGGAGCCCCGTGTTGTGTGTAAGCCGGGATATGTTATCAATGAGGGAATCCCT
GCCTGTGTTCTGCGATCCGATTGCCAAAAGATGTCGTTTCGAAAGGAAGATATGGTACTCGGTGTAACGAACTTTAAGTG
CTTTAGCAGAAATTACGTGTGTAGGAAT-----TTA-----
-----
>Dere
ATGACGAAAACGTGCACCTGGAAGATCTGTGCCCTGCTGGGCCCTCCTATTCTTTTATAAACCCATCGAATCTAGGCGAAT
TGAC-----
TGTACGGCCAACGGAATATGATGCATTGTCTACAGCGTGTCTGAAACCTGCGAGTACTCTGGTATTGGACCCTGCAT
AAGAATGTGCGGAGGCCCTTGTGTGTGTAAGCCGGGATATGTAATCAATGAGAGGATTCGGCTTGTGTTTTCGATCAG
ATTGCCCCAAAGATGTGGTTCCAAGGGAATATATGATTTGGGGAGTACATAATTTTTCATGCTTTGGCGCAAGTAACATG
TGCTTGTACCAGCCAGAGCCTACTGGAAGTCAGAACCACACGTCACCCGCAGATACAGTCGTGACCAT
```

Appendix 2: Genotype counts and inferred allele frequencies at generations 6 and 8.

Gen	Rep	Treatment	Hom_wt	Het	Hom_null	P	q	n
6	1	NS	0	6	6	0.25	0.75	12
6	1	Nx	0	9	3	0.38	0.63	12
6	1	xS	6	5	1	0.71	0.29	12
6	1	xx	5	4	3	0.58	0.42	12
6	2	NS	5	4	2	0.64	0.36	11
6	2	Nx	1	6	5	0.33	0.67	12
6	2	xS	7	4	1	0.75	0.25	12
6	2	xx	2	6	4	0.42	0.58	12
6	3	NS	5	4	3	0.58	0.42	12
6	3	Nx	0	6	6	0.25	0.75	12
6	3	xS	3	7	2	0.54	0.46	12
6	3	xx	4	6	2	0.58	0.42	12
8	1	NS	4	4	5	0.46	0.54	13
8	1	Nx	4	8	2	0.57	0.43	14
8	1	xS	5	7	3	0.57	0.43	15
8	1	xx	4	7	1	0.63	0.38	12
8	2	NS	1	4	10	0.20	0.80	15
8	2	Nx	1	7	7	0.30	0.70	15
8	2	xS	6	8	1	0.67	0.33	15
8	2	xx	6	5	4	0.57	0.43	15
8	3	NS	3	9	1	0.58	0.42	13
8	3	Nx	2	4	9	0.27	0.73	15
8	3	xS	1	8	3	0.42	0.58	12
8	3	xx	6	5	1	0.71	0.29	12