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Female Influence on Pre- and Post-copulatory Sexual Selection and its Genetic Basis in *Drosophila melanogaster*

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Abstract

Genetic variation among females is likely to influence the outcome of both pre- and post-copulatory sexual selection in *Drosophila melanogaster*. Here we use association testing to survey natural variation in 10 candidate female genes for their effects on female reproduction. Females from 91 chromosome 2 substitution lines were scored for phenotypes affecting pre- and post-copulatory sexual selection such as mating and remating rate, propensity to use sperm from the second male to mate, and measures of fertility. There were significant genetic contributions to phenotypic variation for all the traits measured. Resequencing of the 10 candidate genes in the 91 lines yielded 68 nonsynonymous polymorphisms which were tested for associations with the measured phenotypes. Twelve significant associations (markerwise $P < 0.01$) were identified. Polymorphisms in the putative serine protease homolog *CG9897* and the putative odorant binding protein *CG11797* associated with female propensity to remate and met an experimentwise significance of $P < 0.05$. Several other associations, including those impacting both fertility and female remating rate suggest that sperm storage might be an important factor mitigating female influence on sexual selection.

Keywords

Sperm competition; association testing; female mating; sexual selection; genotype-phenotype

Introduction

Sexual selection can be a complex interplay of male and female influence on traits such as mating, sperm utilization and allocation of resources to current versus future matings (Zeh & Zeh 2003). The disparity between the reproductive interests of males and females can potentially lead to sexual conflict as each sex attempts to maximize its own reproductive fitness at the potential cost to members of the opposite sex (Chapman *et al.* 2003a; Parker 2006). Understanding how natural selection might be affecting the evolution of phenotypes influencing sexual selection requires a detailed understanding of patterns of phenotypic variation in natural populations and ideally, knowledge of the genetic polymorphisms underlying the observed variation.

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Data Accessibility

Phenotype data is available as Supplemental Table 2. DNA sequences are available under the Genbank accession numbers JN162918-JN163851 and the genotype file is available as Supplemental Table 3.

In *Drosophila melanogaster*, females frequently mate with and store the sperm from multiple males (Milkman & Zeitler 1974; Prout & Bundgaard 1977; Harshman & Clark 1998; Imhof *et al.* 1998) establishing the opportunity for both pre- and post-copulatory sexual selection to operate. The male perspective and his influence on traits such as induced fidelity and sperm competition has been studied in some detail. We know that a variety of male seminal fluid proteins play a large role in mediating traits affecting sexual selection (Ravi Ram *et al.* 2005; Wolfner 2009) including sperm storage (Wong *et al.* 2008), female fidelity (Chapman *et al.* 2003b; Liu & Kubli 2003; Ram & Wolfner 2009), female egg laying (Herndon & Wolfner 1995) and egg hatching rates (Chapman *et al.* 2001). Furthermore, natural variation in these genes is associated with sperm competition phenotypes (Clark *et al.* 1995; Fiumera *et al.* 2005, 2006, 2007) suggesting that sexual selection might be driving the non-neutral patterns of evolution observed in many male reproductive genes (Clark *et al.* 2006).

Females are also active participants in pre- and post-copulatory sexual selection and thus can be contributing to the variation present in natural populations. Male *Drosophila melanogaster* cannot force copulations on adult females, and several factors are suspected to influence female mating and remating rates. It is well known that the number of sperm in storage has a large effect on female remating rate, the so called ‘sperm effect’ (Manning 1962). This is influenced by the male (Chapman *et al.* 2003b; Liu & Kubli 2003) but could easily be affected by the female as well. For example, sex peptide is gradually cleaved from sperm within the female storage organs, allowing for long-term remating suppression (Peng *et al.* 2005) and female variation in the storage environment might impact the duration of this effect. Females are capable of dumping sperm from storage, which could also play a role in regulating remating rates and sperm utilization (Snook & Hosken 2004; Manier *et al.* 2010). The physical environment also impacts remating rates. Females with reduced access to food are less likely to remate, although this effect disappears once their stored sperm are depleted (Harshman *et al.* 1988). Nutrition also impacts remating rate via differential response to sex peptide (Fricke *et al.* 2009). Density appears to affect female remating rate as well (Gromko & Gerhart 1984; Marks *et al.* 1988), although this effect may depend on the lines that are surveyed (Harshman *et al.* 1988). The perceived attractiveness of the males can also influence mating and remating rates (Jones & Ratterman 2009); females prefer to mate with larger males (Ewing 1961; Wilkinson 1987; Taylor & Kekic 1988; Pitnick 1991; Pitnick & Garcia-Gonzalez 2002; Friberg & Arnqvist 2003) and this may be driven to some degree by environmental factors (Zhang *et al.* 2008). Chemical communication through cuticular hydrocarbons and olfactory receptors has been shown to be important for species recognition (Billeter *et al.* 2009). Although their effects within species are still debated (Takahashi & Ting 2004; Coyne & Elwyn 2006; Greenberg *et al.* 2006), they could be influencing female mate choice in this species.

Currently we have only a cursory characterization of patterns of genetic variation for the female role in pre- and post-copulatory sexual selection in *D. melanogaster*. Clark and Begun (1998) showed that female genotype affects both remating frequency and also the propensity of doubly mated females to use sperm from either the first or second male. Clark *et al.* (1999) then demonstrated a strong interaction between the male and female genotype impacting sperm utilization. Females are known to vary in their ability to resist the cost of mating and much of this variation is due to differences in mating rates (Wigby & Chapman 2004; Linder & Rice 2005). Several genes such as *sarah* (Ejima *et al.* 2004), *muscleblind* (Juni & Yamamoto 2009), *dissatisfaction* (Finley *et al.* 1998), *lozenge* (Fuyama 1995), and insulin signaling genes (Wigby *et al.* 2010), among others, are known to impact female mating. Recently, the receptor for sex peptide, *CG16752*, has been identified and shown to affect female receptivity (Yapici *et al.* 2008), and potentially mediate male × female mating interactions (Chow *et al.* 2010). Furthermore, QTL mapping has identified additional

regions of the genome that might be influential (Lawniczak & Begun 2005). Using an evolutionary EST approach, studies have identified novel genes expressed in the female reproductive tract (Swanson *et al.* 2004; Kelleher *et al.* 2007; Prokupek *et al.* 2008; Kelleher & Pennington 2009; Prokupek *et al.* 2009). Several studies have also used microarray data to compare gene expression in *D. melanogaster* females in response to some aspect of mating such as the transfer of sperm, seminal fluid proteins, and/or courtship (Lawniczak & Begun 2004; McGraw *et al.* 2004; Mack *et al.* 2006; McGraw *et al.* 2008). Overall, we now have a sufficiently strong set of candidate genes to directly investigate the impact of polymorphism on variation among female reproductive fitness components.

Here, we characterized females from 91 second chromosome extraction lines for traits influencing sexual selection, including mating and remating rates, fertility and sperm utilization. We sequenced 10 candidate genes which have been shown to change in expression level due to some aspect of mating (McGraw *et al.* 2004) or are expressed in the female reproductive tract and are likely under positive selection (Swanson *et al.* 2004). In particular, we targeted odorant binding proteins, based on an a priori assumption that they would affect mating decisions. We then tested for associations between genotype and phenotype. We identified several associations between nonsynonymous polymorphisms and phenotypes affecting the female role in sexual selection. Two of the associations, both influencing female remating rate, met an experimentwise P-value < 0.05.

Materials and methods

Scoring phenotypes

Females were selected from 91 chromosome 2 substitution lines originating from a natural *Drosophila melanogaster* population in State College, Pennsylvania (Lazzaro *et al.* 2004). Each homozygous line has a unique second chromosome from nature but they have identical first, third, and fourth chromosomes. The first males to mate were Oregon-R (wild type red eyes) and the second males had a brown dominant (bw^D) eye color mutation. All fly stocks were maintained at medium density on standard agar-dextrose-yeast media at room temperature (~22°C) on a 12 hour light/dark cycle with partially overlapping generations.

Virgin males and females were collected over CO_2, and maintained in single sex vials of five flies until 4–7 days old. Single pair matings of a female from a chromosome extraction line and a single Oregon-R male were set up on day 1 in vial 1 (V1) starting at 0800 hours. Vials were observed for mating at no greater than 15 minute intervals until 1230 hours and the mating times were recorded to the nearest 15 minutes. Males were removed quickly after mating was completed in order to prevent additional copulations. Vials in which no mating was observed were left to mate unobserved until 1900 hours, at which time males were removed. On day 3, females were tapped into vial 2 (V2) with a pair of virgin bw^D males starting at 0800 hours. Matings were observed as described above and all females were tapped to vial 3 (V3) at 1900 hours. On day 8, females were transferred to vial 4 (V4) and then discarded on day 12. Paternity was scored via progeny eye color approximately 16 days after the female was removed from each vial, such that all progeny had an opportunity to eclose. For each of the 91 lines, 10 replicate females were scored in each of two generations (blocks). Only those females that survived the entire experiment were included in the analyses.

The following phenotypes were analyzed: the proportion of virgin females mating within 30 minutes (*mated-30*), the proportion of females that remated (*remated*), female fertility in vial 1 (*fertility-V1*) using only those females that ultimately mated to both males, overall fertility of doubly mated females (*fertility*), and sperm utilization measured as the proportion of offspring sired by the second male to mate (*P2*). Analysis of variance (ANOVA) was used

to test for significance of *fertility-V1*, *fertility* and *P2* between lines (these phenotypes approximated normal distributions). Permutation tests based on chi-squared statistics were used to test for significance of *mated-30* and *remated* (Fiumera *et al.* 2005). Line means were then used for association testing as described below.

Genotyping

PCR amplicons of 10 candidate genes from each line were sequenced (*CG4847*, *CG5395*, *CG6641*, *CG8965*, *CG9820*, *CG9897*, *CG10363*, *CG11797*, *CG13873*, *CG13939*, Table 1). These genes were chosen either because their regulation was altered by some aspect of mating (McGraw *et al.* 2004), or they are expressed in the female reproductive tract and are likely under positive selection (Swanson *et al.* 2004). We focused specifically on odorant binding proteins, under the *a priori* assumption that they would be relevant to mating decisions. PCR and sequencing primers were designed using Primer3 (Rozen & Skaletsky 2000) and we attempted to include ~1 kb upstream and downstream of the coding region. All sequencing was completed using Applied Biosystems Automated 3730 DNA Analyzer with Big Dye Terminator chemistry according to manufacturers' protocols. Sequences were clipped to maximize regions with error rates below 0.01 using the program CodonCode Aligner (www.codoncode.com), and were then assembled by line into contigs. All sequences with fewer than 25 bases were discarded, as were sequences with fewer than 50 Phred20 bases. Contigs were aligned to reference sequences obtained from flybase.org. Polymorphism tables were exported from CodonCode Aligner.

Association testing

Association tests between genotype and phenotype were conducted in MATLAB using permutation tests based on simple linear regression. We tested the effects of nonsynonymous polymorphisms, synonymous polymorphisms and non-coding polymorphisms independently. For each phenotype, we used 100,000 permutations based on the F-value from the linear model to generate both markerwise and experimentwise *P*-values (Churchill & Doerge 1994). Experimentwise *P*-values may represent a conservative statistic, as they compare observed *P*-values to permuted values across all polymorphisms. Markerwise *P*-values are less stringent, and do not adequately control for the large number of tests completed. In order to correct for this, we also applied a False Discovery Rate calculation (FDR) to the markerwise *P*-values to control for false positives (Storey & Tibshirani 2003).

Results

A total of 1686 females were scored for propensity to mate within 30 minutes and propensity to remate. 1554 of these females mated to a second male in vial 2, and were scored for fertility in vial 1, overall fertility, and sperm utilization. There were highly significant line effects ($P < 0.005$) for all the traits scored (Table 2) but no significant effect of block (not shown). Fertility in vial 1 (*fertility-V1*) averaged 20.7 offspring and line means ranged from 1.8 to 39.5 offspring, while overall fertility averaged 85.8 offspring and lines means ranged from 39.4 to 137.8 offspring. Overall, approximately 56% of virgin females mated within 30 minutes but line means ranged from 6% up to 88%. On average, 48% of the females ultimately remated to the second male and line means ranged from 7% to 94%. Among those females that doubly mated, approximately 56% of the offspring were sired by the second male to mate and line means ranged from 8% to 89%. *Fertility* and *fertility-V1* were strongly positively correlated ($r = 0.681$, $P < 0.001$) which is not surprising given one is a subset of the other. In addition, *P2* and *fertility* were negatively correlated ($r = -0.22$, $P = 0.039$). We further analyzed this finding by decomposing fertility into fertility in the first and second vials (*fertility*-V1V2) and fertility in the third and fourth vials (*fertility-V3V4*), such that *fertility-V1V2* corresponds almost entirely to progeny of the first male, and

fertility-V3V4 corresponds to progeny from both the first and second male. *Fertility-V1V2* and *fertility-V3V4* were positively correlated (r = 0.576, P < 0.001). *Fertility-V3V4* was strongly negatively correlated with *P2* (r = −0.272, *P* = 0.009) but *fertility-V1V2* was not correlated with *P2* (r = −0.040, *P* = 0.708). Females that tended to use the second male's sperm also tended to have reduced overall fertility and this was due to reduced fertility after the second mating.

The analyzed genes were highly polymorphic (Table 1). We excluded 494 singletons and 6 polymorphisms with less than 8 lines successfully scored. This left 68 nonsynonymous polymorphisms, 157 synonymous polymorphisms and 746 polymorphisms in noncoding regions. The pattern of linkage disequilibrium among the 68 nonsynonymous polymorphisms is shown in Figure 1.

Association Testing

We identified twelve (12) significant associations (markerwise $P < 0.01$; q-value = 0.22) at nonsynonymous polymorphisms. These associations included five different genes and four phenotypes. Associations were identified between *mated-30* and *CG9820*, *remated* and *CG9820*, *CG9897* (4 polymorphisms), *CG10363* and *CG11797* (2 polymorphisms), *fertility-V1* and *CG9897* and *CG10363*, and *fertility* and *CG11797* (Table 3). Some of these markers are in high linkage disequilibrium (Figure 1) with each other and therefore may not actually represent 12 independent effects.

Two of these associations were significant at an experimentwise $P < 0.05$ (Figure 2), both affected the likelihood a female would remate (*remated*) and interestingly both were in regions with several linked amino acid polymorphisms. The first is an isoleucine to asparagine change at position 88 of the serine protease homolog, *CG9897*. This polymorphism was flanked by 3 other nonsynonymous changes all within 13 amino acids; arginine to serine at position 76, glycine to aspartic acid at position 83, and alanine to serine at position 89. All of these nonsynonymous polymorphisms associated at markerwise $P < 0.005$ although a synonymous change within this region did not ($P = 0.59$). Because these polymorphisms are in linkage disequilibrium and closely linked, we attempted to parse the individual effects by examining all four polymorphisms simultaneously using an ANOVA model. The isoleucine to asparagine polymorphism at position 88 and the alanine to serine polymorphism at position 89 retained significance at $P < 0.05$. Using both of these markers to form a haplotype also resulted in a significant association ($P = 1.9 \times 10^{-5}$) and all pair wise comparisons between the three different haplotypes were significant (Figure 2A). The other experimentwise association with remating rate is an alanine to valine change at position 32 of the putative odorant binding protein *CG11797* (Figure 2B). Interestingly, there is a lysine to a premature stop codon at position 33, just one amino acid downstream. Both of these nonsynonymous polymorphisms were associated at a markerwise $P < 0.005$. When both were included in an ANOVA model only the alanine to valine change at position 32 had a significant effect ($P < 0.05$), the premature stop codon did not.

Several genes showed evidence of pleiotropy (Table 3). The two genes mentioned above, *CG9897* and *CG11797* also associated with *fertility-V1* and *fertility*, respectively. Two different nonsynonymous polymorphisms in the putative olfactory receptor *CG9820* (Lys31Asn and Glu113Lysin) associated with female mating when the females were virgins (*mated-30*) and after they had already mated once (*remated*), respectively. Finally, a lysine to proline change at position 955 in the putative peptidase inhibitor *CG10363* was associated with *fertility-V1*, while an aspartic acid to glutamic acid change at position 1491 was associated with female remating rate (*remated*).

Among synonymous and noncoding polymorphisms, even the most significant associations (markerwise $P < 0.005$) still had a false discovery rate of around q = 0.5. These can be viewed in Supplementary Table 1. Although half these associations could be biologically relevant, the nonsynonymous polymorphisms highlighted above show the most promise to be causative or linked to the traits of interest. All line means were normally distributed except for *mated-30* (*P=0.019*), which showed some evidence of a bimodal distribution.

Discussion

Here we used association testing to survey natural variation and study the genetic basis to female influence on pre- and post-copulatory sexual selection in *D. melanogaster*. We sequenced 10 candidate genes in 91 chromosome extraction lines and scored females from these lines for mating rate, remating rate, propensity to use the sperm from the second male and fertility. There was a significant genetic basis to variation for all the traits studied. By independently testing nonsynonymous polymorphisms, we identified two associations with remating rate that met a stringent experimentwise $P < 0.05$ (*CG9897* and *CG11797*) while 10 additional associations (across a variety of genes and phenotypes) met a more liberal markerwise $P < 0.01$.

CG9897 is a putative serine endopeptidase homolog (Ross *et al.* 2003). It was identified as a candidate gene due to its expression in female reproductive tracts (Swanson *et al.* 2004), and recent work demonstrates that it is expressed in the sperm storage organs (Prokupek *et al.* 2009). An isoleucine to asparagine change at position 88 of *CG9897* associated with female remating rate (experimentwise $P < 0.05$). Three other nonsynonymous polymorphisms in this gene (at positions 76, 83 and 89) were also associated with remating rate at a markerwise $P < 0.01$. When considering these markers in an ANOVA simultaneously, only the polymorphisms at amino acid positions 88 and 89 remained significant, but it is interesting to observe so many amino acid polymorphisms in this small region. Although speculative, the expression of *CG9897* in the sperm storage organs may suggest some influence on how sperm behaves in storage, and thus relate to the so called ‘sperm effect’, whereby females without properly stored sperm are more likely to remate (Manning 1962). We also identified an association between a glycine to aspartic acid change at position 83 in *CG9897* and female fertility (markerwise $P < 0.01$). This could also be driven through an effect on sperm storage but could also be the result of pleiotropy as we do not see a correlation between female fertility and remating rate as might be expected if both were being driven via this gene’s impact on sperm storage. Interestingly, *CG9897* shows evidence for elevated levels of DNA polymorphism and signatures of balancing selection (Panhuis & Swanson 2006) and may be interacting with male reproductive genes that are also known to exhibit high levels of polymorphism (Swanson & Vacquier 2002; Clark *et al.* 2006). Serine endopeptidase homologs are thought to lack proteolytic activity, but have been implicated in mediating protein interactions and immune responses (Kawabata *et al.* 1996; Asgari *et al.* 2003; Ross *et al.* 2003; Yu *et al.* 2003). Clearly, more work needs to be done to understand the mechanisms and selective forces acting on *CG9897*, but association tests in another population support these findings and analysis of the RNAi knockdown is ongoing (Chow, Wolfner and Clark, unpublished data).

The second association meeting the experimentwise threshold was an alanine to valine polymorphism at position 32 of *CG11797*. A lysine to premature stop codon at position 33 also was associated (markerwise $P < 0.005$), but this association was not significant in an ANOVA model testing the simultaneous effects of both markers. *CG11797*, also known as *Obp56a*, codes for an odorant binding protein. *D. melanogaster* encode 51 odorant binding proteins, which aid in the solution and transfer of odorants to specific receptors (Hekmat-Scafe *et al.* 2002). These odorant binding proteins are potentially of great interest in the

context of female mating decisions. Species and gender recognition in *D. melanogaster* rely on both the production and assessment of cuticular hydrocarbons (Billeter *et al.* 2009; Lacaille *et al.* 2009). Changes in cuticular hydrocarbon profiles, specifically polymorphism at the desaturase 2 gene, have also been implicated in sexual isolation (Fang *et al.* 2002; Greenberg *et al.* 2003). These results suggest that polymorphisms in odorant binding proteins or odorant receptors may play a role in female mating decisions. Odorant binding proteins are particularly noteworthy, as they have recently been found among male seminal fluid proteins, indicating a possible role in male induced post-copulatory phenotypes in addition to female pre-copulatory decision making (Findlay *et al.* 2008). Interestingly, *CG11797* is likely under positive selection, one of only two genes in the family to show this pattern of selection (Wang *et al.* 2007). A threonine to alanine polymorphism at position 15 in *CG11797* was also associated with female fertility (markerwise $P < 0.01$). As both *CG9897* and *CG11797* were associated with both fertility and female remating propensity, this raises the possibility that there may be some relationship between these phenotypes, although we do not see a genetic correlation between these phenotypes in the lines assayed ($r = -0.11$, $P = 0.28$).

A lysine to asparagine change at position 31 and a glutamate to lysine change at position 113 in another odorant binding protein, *CG9820*, associated with mating rate when the females were virgins (*mated-30*) and remating rate (*remated*), respectively. Although *CG9820* appears to affect both mating and remating rates, no single polymorphism was associated with both traits and we did not observe a genetic correlation between these phenotypes ($r = 0.14$, $P = 0.18$). It is interesting to note that mating rate when the females are virgins appears to be independent of the propensity of these females to remate. It would be very exciting if these two traits were independently controlled, but our observation could be generated by a strong male by female interaction (Clark *et al.* 1999; Chow *et al.* 2010). Under such a scenario, the interaction term between the male and female genotype swamps the marginal effects of the female genotype when scoring mating and remating rate using only two different male genotypes. Although it would demand substantial added effort, a common genetic basis underlying mating and remating rate might be observed if this experiment were repeated using the same males but in the reciprocal order.

Surprisingly, we did not identify associations for a female's propensity to use sperm from the second male, despite significant differences in *P2* among the surveyed lines in this study ($P = 0.005$) and previous evidence that female genotype influences sperm utilization (Clark & Begun 1998). It is possible that we lack the statistical power to identify true associations because many genes of very small effect impact *P2*. It is also possible that the ten genes we chose to survey may not be important in regulating sperm utilization patterns. We did not observe associations with any of the noncoding or synonymous polymorphisms that we scored; even the most significant of these yielded a false discovery rate of about 50% (see supplemental table 1). This is surprising since some of these genes were selected based on changes in expression level after mating (McGraw *et al.* 2004). The lack of association may be due to the large number of tests (and the requirement to control for these tests) that were completed for noncoding and synonymous polymorphisms as compared to the nonsynonymous changes. It would be interesting to look more closely at the effects of polymorphism within promoter regions for these genes, but these regions are not yet well defined.

In addition to the correlations between fertility measures, we observed a negative relationship between *fertility* and the propensity of a female to use sperm from the second male (*P2*). One explanation for this correlation between *P2* and *fertility* is variation in male quality. Females are known to vary in the extent to which they use sperm from the second male to mate (Clark & Begun 1998). If certain female genotypes are inclined to use sperm

from the second male regardless of quality, via mechanisms such as sperm dumping or ejection (Snook & Hosken 2004; Manier *et al.* 2010), she may find herself in the unfortunate position of reducing her overall fertility in the instance of a low quality second mating partner. To explore this possibility, we further decomposed fertility into fertility in vials 1 and 2 (*fertility-V1V2*) and fertility in vials 3 and 4 (*fertility-V3V4*), which are positively correlated. We found that *fertility–V1V2*, when the female had very little chance to lay eggs sired by the second male, was uncorrelated with *P2* ($r = -0.040$, $P = 0.708$) however *fertility-V3V4* showed a strong negative correlation with *P2* ($r = -0.272$, $P = 0.009$). This is consistent with the hypothesis of sperm dumping by the female coupled with mating to a low quality second partner.

The negative correlation between *P2* and *fertility* could also be explained by effects of variation in female reproductive tract environment on sperm longevity. Under this scenario, female genotypes causing sperm to degrade in quality quickly could result in reduced hatchability of eggs sired by the first male with increasing time after mating. If the female continues laying eggs sired by the first male despite this loss of hatchability, this would lead to an inflated *P2* value and could also lead to decreased fertility. Previous work has identified an effect of male genotype on sperm longevity in storage (Chapman *et al.* 2001; Civetta *et al.* 2008) and has shown that seminal fluid has a protective effect on sperm in the female reproductive tract (Holman 2009), but no effect of female genotype on sperm longevity was identified. However, this does not preclude such a possibility, as neither study attempted to assess variation across a wide variety of female backgrounds. As mentioned above with the effect of *CG9897* on remating rate, it is possible that much of the variation in female influence on postcopulatory sexual selection is driven through sperm storage (or ejection) rates, a potentially productive area for future research.

Another important problem is to quantify the degree to which the female influence on postcopulatory sexual selection impacts her overall fitness. The effects of female fertility are obvious, but the fitness benefits of female choice remain an active area of research (Jennions & Petrie 2000; Zeh & Zeh 2003; Hettyey *et al.* 2010). Understanding the benefits of female choice can be further complicated when there are strong male by female interactions (Clark *et al.* 1999) potentially influencing parentage through cryptic female choice (Eberhard 1996). Females do have some control over how sperm is utilized (Qazi & Hogdal 2010), although much of the observed dynamics may fit a ‘fair raffle’ (Manier *et al.* 2010). Further research characterizing the fitness consequences of female influence on postcopulatory sexual selection will greatly enhance our understanding of reproductive outcomes.

Caveats of association testing

It is important to recognize that association testing does not imply causality and the potential for false positives is well recognized (Cowperthwaite *et al.* 2010). By focusing on nonsynonymous polymorphisms, we identified twelve significant associations between genotype and phenotype at a markerwise $P < 0.01$ and a false discovery rate calculation using Q-value (Storey & Tibshirani 2003) suggests that perhaps two of these associations are false positives. Several factors, however, indicate that false positives are not the sole driving force underlying the identified associations. First, the genes in this study were selected *a priori* based on the biological assumption that they could be affecting female influence on sexual selection. We also tested for associations between our female phenotypes and polymorphisms in male reproductive genes (Fiumera *et al.* 2005) or immunity genes (Lazzaro *et al.* 2004) that had been genotyped in these same lines. No markers from these data sets met an experimentwise $P < 0.05$. In addition, female reproductive genes had significantly more associations meeting a markerwise $P < 0.01$ as compared to either male reproductive genes ($P = 0.007$) or immunity genes ($P = 0.015$) indicating that long distance, unobserved linkage disequilibrium is not driving the observed

associations. Furthermore, we observed the strongest associations with amino acid polymorphisms as compared to synonymous or arbitrary noncoding polymorphisms that were identified.

In *D. melanogaster*, and other non-human species, the goal of association testing may not be to identify markers for diagnostics but to provide a detailed genetic screen that can inform future studies using technologies such as RNAi. For example, Fiumera *et al*. (2005) identified a weak association between *Acp29Ab* and the proportion of offspring sired by the first male to mate and this was subsequently verified using a null mutation (Wong *et al.* 2008). As such, it is often acceptable to allow a slightly liberal false discovery rate to prevent missing true associations. The synonymous and noncoding associations had much higher false discovery rates; the Q-value was greater than 0.5 for associations with a markerwise $P < 0.005$. This makes it less likely that these represent true associations (Supplemental Table 1), but these results certainly motivate direct testing of RNAi knockdowns to assess their impact on the phenotypes measured here.

In summary, we have shown that extensive genetic variation exists for female influence on postcopulatory sexual in *D. melanogaster*. Furthermore, we have used association testing to identify twelve associations of polymorphisms in four genes with mating rate, remating rate and measures of female fertility. Two different genes related to olfactory systems influence either mating or remating rate suggesting that pheromonal communication may be important within this species. In addition, a serine protease homolog associated with both fertility and remating rate and this may be influencing sperm storage or ejection. If, as we believe, these genes are crucial determinants of female post-mating behavior, then further research may answer questions about the nature of antagonistic sexual coevolution in *D. melanogaster*, and help to explain the dynamics of male × female interactions in determining mating outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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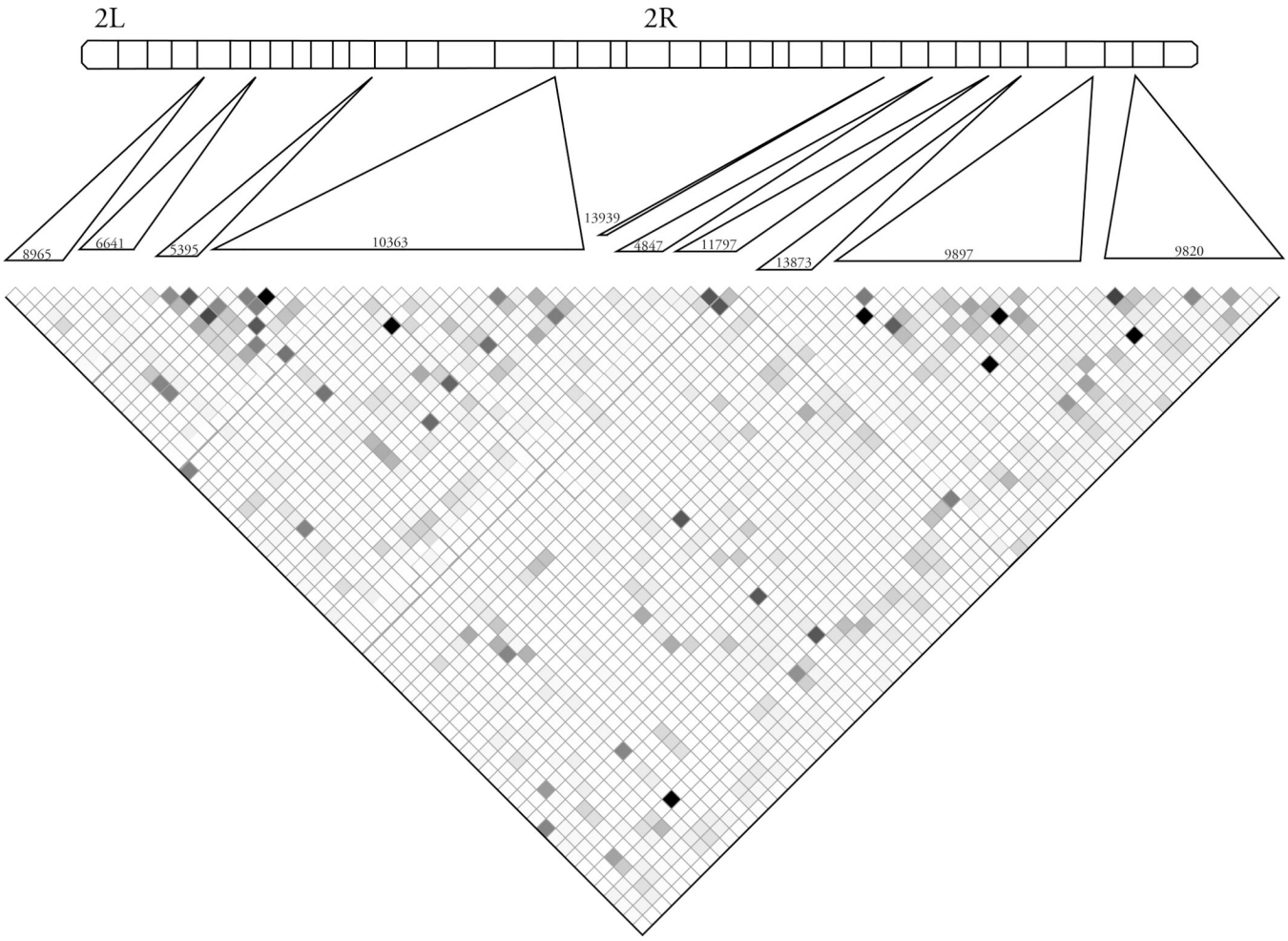

Figure 1.
Patterns of linkage disequilibrium for the nonsynonymous polymorphisms. Markers are arranged in order along chromosome 2 and the approximate location of each gene on chromosome 2 is shown. Darker colors indicate higher levels of linkage disequilibrium (r^2 = 1 shown in black, $1 > r^2 > 0$ shown with shades of gray, $r^2 = 0$ shown in white).

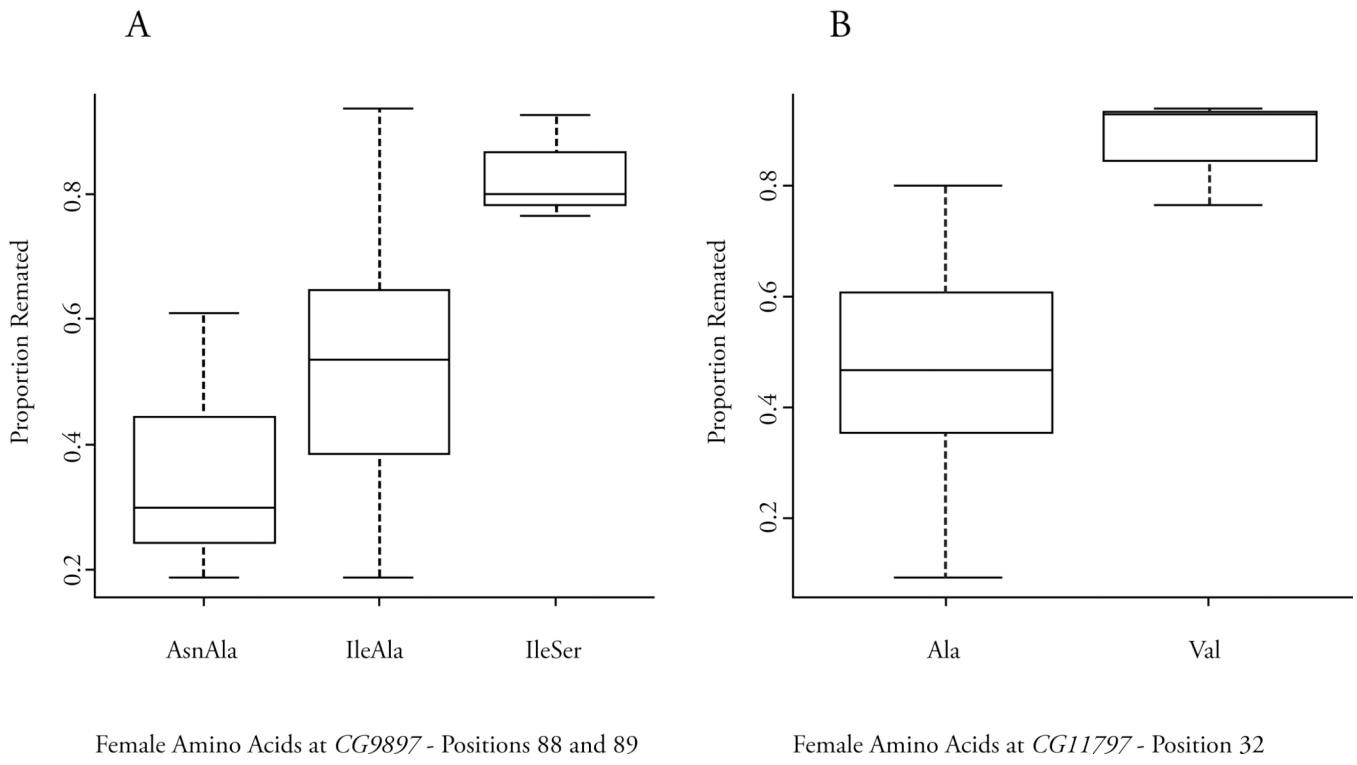

Figure 2.
Examples of associations with female influence on sexual selection. Box plots showing the median, upper and lower quartiles and the range for the different amino acid polymorphisms that associate with the proportion of females that remated at *CG9897* (A) *and CG11797* (B).

Table 1

Summary of the genes analyzed. The putative function according to FlyBase, the number of nonsynonymous polymorphisms (d_N), the number of synonymous polymorphisms (d_S) and the number of noncoding polymorphisms (*noncoding*) that were identified.

Gene	Putative Function	d_S	d_N	*noncoding*
CG4847[c]	endopeptidase	15	4	97
CG5395[c]	ATPase activity	8	3	32
CG6641[a]	pheromone binding	4	4	47
CG8965[c]	unknown	33	4	115
CG9820[a]	olfactory receptor	22	10	81
CG9897[b]	endopeptidase	17	14	103
CG10363[b]	peptidase inhibitor	26	20	44
CG11797[a]	odorant binding	6	4	80
CG13873[a]	odorant binding	8	4	100
CG13939[a]	odorant binding	18	1	47
	Total	157	68	746

Genes were identified from McGraw et al. 2004[a], Swanson et al. 2004[b] or both[c]

Table 2

Summary of phenotypic distributions.

Phenotype	Mean of Line Means	Range of Line Means	Standard Error for Line Means	Statistic	P-value
Fertility-V1	20.66	1.8 – 39.5	0.87	$F_{90,549} = 3.32$	$P < 0.0001$
Fertility-overall	85.76	34.9 – 137.8	2.69	$F_{90,549} = 3.27$	$P < 0.0001$
Mated-30	0.56	0.06 – 0.88	0.02	$X^2 = 91.42$	$P < 0.0001$[a]
Remating	0.48	0.07 – 0.94	0.02	$X^2 = 99.75$	$P < 0.0001$[a]
P2	0.56	0.08 – 0.89	0.02	$F_{90,549} = 1.48$	$P = 0.005$

[a] *P*-value determined via permutation

Table 3

Associations with nonsynonymous polymorphisms. Shown is the gene with the location of the polymorphism.

Phenotype	Gene (snp)	Polymorphism	r^2
Mated-30	*CG9820*	Lys31Asn*	0.08
Remated	*CG9820*	Glu113Lys**	0.11
	CG9897	Arg76Ser**, *a*	0.15
	CG9897	Gly83Asp**, *a*	0.14
	CG9897	Ile88Asn***	0.19
	CG9897	Ala89Ser**	0.16
	CG10363	Asp1491Glu*	0.08
	CG11797	Ala32Val***	0.17
	CG11797	Lys33Stop**, *a*	0.09
Fertility-V1	CG9897	Gly83Asp**	0.10
	CG10363	Leu955Pro**	0.11
Fertility	CG11797	Thr15Ala*	0.09

*** experimentwise $P < 0.05$,

** markerwise $P < 0.005$,

* markerwise $P < 0.01$

a no longer significant ($P < 0.05$) after inclusion of linked markers into ANOVA.