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Genetic variation but weak genetic covariation between pre- and post-copulatory episodes of sexual selection in *Drosophila melanogaster*

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Abstract

When females mate polyandrously, male reproductive success depends both on the male's ability to attain matings and on his ability to outcompete rival males in the fertilization of ova post-copulation. Increased investment in ejaculate components may trade off with investment in precopulatory traits due to resource allocation. Alternatively, pre- and post-copulatory traits could be positively related if individuals can afford to invest heavily in traits advantageous at both episodes of selection. There is empirical evidence for both positive and negative associations between pre- and post-copulatory episodes, but little is known about the genetic basis of these correlations. In this study, we measured morphological, chemical and behavioural precopulatory male traits and investigated their relationship with measures of male fitness (male mating success, remating inhibition and offensive sperm competitiveness) across 40 isofemale lines of *Drosophila melanogaster*. We found significant variation among isofemale lines, indicating a genetic basis for most of the traits investigated. However, we found weak evidence for genetic correlations between precopulatory traits and our indices of male fitness. Moreover, pre- and post-copulatory episodes of selection were uncorrelated, suggesting selection may act independently at the different episodes to maximize male reproductive success.

Introduction

Sexual selection arises because of differential reproductive success due to competition for mates and their gametes, and is often more intense in males as their fitness depends more on mating success than does female fitness (Darwin, 1871; Bateman, 1948). An important consequence of sexual selection on males is the evolution of rapidly diverging traits (i.e. ornaments and weapons) that provide an advantage to males in gaining matings (Andersson, 1994). However, there has been an increasing awareness over the past few decades of the evolutionary importance of post-copulatory mechanisms (Parker, 1970a; Eberhard, 1996; Simmons, 2001; Birkhead & Pizzari, 2002). Due to the ubiquity of multiple mating by females (Simmons, 2005; Kvarnemo & Simmons, 2013), it is now recognized that sexual selection can extend beyond mating. Consequently, male reproductive success is not only determined by a male's ability to obtain matings but also the ability to fertilize ova after mating through sperm competition (Parker, 1970a; Simmons, 2001) and/or cryptic female choice (Eberhard, 1996). Post-copulatory processes can produce substantial variation in paternity among males and have the potential to reinforce or counteract the effects of precopulatory selection (Collet *et al.*, 2012; Kvarnemo & Simmons, 2013). Hence, all episodes of sexual selection need to be examined to accurately assess male fitness (Lewis *et al.*, 2013). The relationship between pre- and post-copulatory sexual selection has become a major issue because it informs on whether traits are exposed to similar selection pressures during different episodes of selection, and ultimately this relationship determines the net action of sexual selection

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(Arnold & Wade, 1984; Webster *et al.*, 1995; Collet *et al.*, 2012; Pischedda & Rice, 2012; Kvarnemo & Simmons, 2013; Pélissié *et al.*, 2014; Devigili *et al.*, 2015).

Precopulatory selection depends on a male's ability to attain copulations with females, whereas post-copulatory selection reflects differences in siring success among males determined by aspects of ejaculate components and/or sperm utilization by the female. The maintenance and expression of traits in both pre- and post-copulatory episodes of selection is costly (Pitnick, 1996; Emlen, 2001; Simmons *et al.*, 2010). Theory predicts that increasing levels of post-copulatory selection should promote increased male expenditure on sperm production (Parker, 1998). However, theory assumes that males successful at gaining access to mates due to high investment in precopulatory traits should have fewer resources available for investment in post-copulatory traits such as sperm competitiveness (Parker *et al.*, 2013), leading to a negative correlation between traits favoured through pre- vs. post-copulatory sexual selection (Simmons & Emlen, 2006; Fitzpatrick *et al.*, 2012; Lüpold *et al.*, 2014; Dines *et al.*, 2015). An increasing number of studies show evidence for trade-offs between pre- and post-copulatory selected traits: males successful in obtaining matings can be limited in their ability to produce costly ejaculates and thus are unable to take advantage of their enhanced mating success (Preston *et al.*, 2001; Bussière *et al.*, 2005; Simmons, 2005; Demary & Lewis, 2007). In bushcrickets, males that mate more often deliver smaller spermatophores than males who mate less frequently (Simmons, 1995). Preston *et al.* (2001) found Soay rams most successful in precopulatory sexual selection sire a lower percentage of offspring towards the end of the mating season due to the effects of sperm depletion. Similarly, Demary & Lewis (2007) found that male fireflies with more attractive bioluminescent displays had lower paternity success, which suggests a possible trade-off between investment in precopulatory courtship display and investment in ejaculates (South & Lewis, 2012).

In contrast, pre- and post-copulatory selection can be aligned if enhanced expression of a precopulatory trait is indicative of genetic quality (Johnstone, 1995), which could lead to greater ability to acquire resources and thus superior ejaculate quality (Rowe & Houle, 1996). When variance in attaining resources exceeds variance in allocation of resources needed for growth and reproduction, high-quality males may be able to invest heavily in both precopulatory traits and ejaculate production, leading to a positive relationship between attractiveness and post-copulatory investment (Kvarnemo & Simmons, 2013). For instance, in *Drosophila melanogaster*, male body size has been identified as a trait favoured by precopulatory sexual selection (Partridge & Farquhar, 1983) and similarly favoured in post-copulatory selection as demonstrated by the correlation between large body size and fertilization success (McLain, 1985; Bangham *et al.*, 2002). In flour beetles, males that produce more attractive olfactory cues have higher paternity share (Lewis & Austad, 1994). Finally, in guppies, males that have high levels of orange pigmentation are more attractive to females and more successful in sperm competition (Evans *et al.*, 2003). These studies thus demonstrate that episodes of pre- and post-copulatory selection can reinforce each other.

The fact that pre- and post-copulatory episodes of sexual selection can be subject to trade-offs or be aligned means that focusing simply on either the pre- or post-copulatory episodes of selection provides an incomplete picture of the action of sexual selection, and that consequently, both episodes must be considered. Phenotypic relationships between traits such as those described above are predicted to be influenced by the availability of resources, with trade-offs expected when resources are limited (Stearns, 1992). However, to understand the evolution of traits subject to sexual selection, we need to discern the underlying genetic variance of and covariance between traits (Roff & Fairbairn, 2007). When traits are paired due to pleiotropy and/or linkage, selection acting on one trait can result in correlated changes in the trait that is genetically coupled with the trait under selection (Lande & Arnold, 1983). A negative covariance between traits favoured in pre- vs. post-copulatory selection will slow the rate of evolutionary change by reducing the strength of directional selection on either trait (Lynch & Walsh, 1998), whereas positive genetic correlations can enhance the evolutionary potential of a given trait (Kvarnemo & Simmons, 2013). Whereas the study of phenotypic relationships between traits determining pre- and post-copulatory components of reproductive success has revealed the existence of negative phenotypic correlations in some cases, these may not necessarily inform on the existence of an underlying genetic trade-off. Only a few studies have investigated the genetic relationships between traits involved in pre- and post-copulatory episodes. For example, in the scorpion fly *Panorpa cognata*, Engqvist (2011) found a significant negative genetic association between attractiveness and mating investment. Hosken *et al.* (2008) found a positive relationship between male attractiveness and offensive fertilization success in *Drosophila simulans*, and in the dung beetle, *Onthophagus taurus*, Simmons & Kotiaho (2002) found a positive genetic correlation between male condition, which determines courtship rate and male mating success, and testis weight and sperm length, which determine paternity success in this species (Garcia-Gonzalez & Simmons, 2007). Investigating patterns of genetic covariance between traits is paramount to understanding evolution through sexual selection, as the direction of these correlations reveals their potential to either promote or constrain coevolutionary responses to selection (Lynch & Walsh, 1998). Here, we examine patterns of genetic

variation and covariation between pre- and post-copulatory traits among isofemale lines of *D. melanogaster* recently derived from the field.

Studies of *D. melanogaster* have played a central role in sexual selection research. With considerable levels of female multiple mating in this species (Imhof *et al.*, 1998; Kuijper & Morrow, 2009; Travers *et al.*, 2015) males face competition both to secure matings and to fertilize ova. Consequently, traits that affect male success in both pre- and post-copulatory episodes of selection have evolved. Male mating success in *D. melanogaster* has been reported to be influenced by secondary sexual traits such as sex combs (Ahuja & Singh, 2008) and cuticular hydrocarbons (CHCs; Ferveur, 2005), as well as body size (Partridge & Farquhar, 1983; Partridge *et al.*, 1987b; Bangham *et al.*, 2002; Jagadeeshan *et al.*, 2015). Male reproductive success is also determined after copulation through sperm competition (Price *et al.*, 1999; Fiumera *et al.*, 2005; Manier *et al.*, 2010; Lüpold *et al.*, 2012) and the action of seminal fluid proteins on female behaviour and physiology (Kalb *et al.*, 1993; Chapman, 2001; Pitnick *et al.*, 2001a; Wigby *et al.*, 2009). Therefore, we examined the relationship between male mating success and a number of precopulatory traits, including morphological (body size, sex comb tooth number), olfactory (CHCs) and behavioural (courtship duration) traits previously shown to be targets of sexual selection on males. Males of this species manipulate female mating rates by transferring seminal fluid proteins that inhibit remating (Scott, 1986; Chen *et al.*, 1988; Aigaki *et al.*, 1991; Kalb *et al.*, 1993; Chapman, 2001; Wolfner, 2002). Given the high last male sperm precedence in this species, inhibition of female remating may be a strong determinant of male reproductive success (Clark *et al.*, 1995). We thus investigated the relationship between precopulatory male traits and measures of male fitness (male mating success, male-induced inhibition of female remating and offensive sperm competitiveness). For clarity, we consider traits that affect mating success as precopulatory traits and those that influence siring success as post-copulatory traits, although we recognize that it is possible for a trait to contribute to both pre- and post-copulatory success where, for example, females choose cryptically sperm from males found most attractive in courtship to fertilize their ova. Finally, we examined the genetic relationship between precopulatory mating success and post-copulatory fertilization success to establish whether post-copulatory sexual selection reinforces or counteracts precopulatory selection, or whether these processes of sexual selection operate independently.

Materials and methods

Drosophila melanogaster isofemale lines

Drosophila melanogaster used in this study were collected from the Margaret River region of Western Australia in April 2013. We established 40 isofemale lines by placing single wild-caught females into individual vials. From thereon, lines were maintained through within line matings by placing approximately 20 individuals from each line into a vial to lay with 10 mL maize food medium. We discarded the adults after 12 h to prevent larval overcrowding and to maintain nonoverlapping generations. Eggs, larvae and adult flies were maintained at 25 °C on a 12L : 12D diurnal cycle throughout all experiments.

All traits were measured between generation 10 and 13 as follows: P_2 (generation 10), egg-to-adult viability (generation 11), inhibition of remating (generation 12). Male mating success, sex comb number and wing area were measured on the same individuals in generation 13. CHCs were also measured at generation 13. To generate experimental flies for each assay, flies from each line were placed in a population cage with a grape agar plate for 4 h. The following day, we collected first instar larvae and transferred them to vials containing 10 mL of sugar-maize medium at a standard density of 40 larvae per vial. We collected offspring 9–11 days later under CO_2 anaesthesia within 8 h of eclosion and transferred males to single sex vials.

Male mating success

We measured male mating success by presenting males with five different tester female genotypes over five consecutive days. For each isofemale line we assessed the mating success of 6.4 ± 1.0 replicate males. We standardized the female genetic background by selecting virgin females from one of five isogenic lines at each mating opportunity (e.g. isogenic line 1 in the first opportunity for all males, isogenic line 2, 3, 4 and 5 for the 2nd, 3rd, 4th and 5th mating opportunity). We used these isogenic tester females (hereafter referred to as tester females) to reduce genetic variation in female mate preference at each mating opportunity (Bjork *et al.*, 2007; Droge-Young *et al.*, 2012) and used tester females from a different isogenic line each day to gain a measure of male attractiveness assessed across a variety of female genotypes. Each tester line was generated through a protocol of full sibling matings started with one founder pair of flies taken from a replica of an LH_M population (see Byrne & Rice, 2005 for details). The tester lines were obtained through a protocol of brother–sister matings for 16 generations, followed by several generations of within line matings (approximately 15 individuals from each vial for each new generation), before reinstating a full sibling mating protocol for another 21 generations. We used 2- to 3-day-old virgin tester females for the mating trials to reduce variation in female preference due to environmental effects (e.g. female age and mating history; Gray, 1999; Moore & Moore, 2001; Judge *et al.*, 2010; L.M. Travers, L.W. Simmons, F. Garcia-Gonzalez,

unpublished manuscript). The mating trials were conducted by placing each male into a vial with a single tester female for 20 min. We recorded courtship duration for pairs that started mating within 20 min. If mating did not commence within 20 min, we removed the female from the vial and gave the male a mating score of 0 for that trial. After the 5 days of mating trials, males were stored at −80 °C for later sex comb and wing size measurements.

Male inhibition of female remating

To measure male ability to inhibit female remating through the action of seminal fluid components (Chen *et al.*, 1988; Aigaki *et al.*, 1991; Kalb *et al.*, 1993), virgin tester females from an isogenic line were mated to a single focal isofemale line male by placing individual pairs into vials and observing for 2 h. We assessed the remating inhibition of 2.9 ± 1.3 males per isofemale line. We used tester females to control for genotypic male × female interactions on female remating rates (Travers *et al.*, 2015). After a mating pair disengaged, males were removed from the vial to prevent remating with the same male. Tester females were then given the opportunity to remate once every 12 h for up to 5 days. Remating opportunities were given by placing a sexually naïve male from one isogenic line (hereafter referred to as tester males) into a female's vial and observed for 2 h. We used tester males from the same isogenic line in each remating opportunity to control for genetic variation in male attractiveness (Kosuda, 1985; Partridge *et al.*, 1985; Taylor *et al.*, 2007). Remating opportunity 1 was presented 12 h after the first mating, and opportunities 2, 3 and 4 were presented 24, 36 and 48 h after the first mating, respectively, with a maximum of 10 opportunities over 5 days. The number of remating opportunities received before a female remated was taken as a measure of her first (focal isofemale line male) mate's ability to inhibit remating. Females that did not remate in the 5 days were given a score of 11 (only seven females).

Offensive sperm competitiveness and egg-to-adult viability

We measured offensive sperm competitiveness (P_2) of 2.8 ± 1.7 males per isofemale line by calculating the proportion of offspring produced when focal males from isofemale lines were mated in the second mating position to a previously mated female. We used tester males from a single isogenic line in the defensive mating position (P_1) across all isofemale lines to control for genotypic male × male interactions and also to control for differences in sperm competitiveness of rival males (Garcia-Gonzalez, 2008b; Garcia-Gonzalez & Evans, 2011; Travers *et al.*, 2016). We also used tester females from a single isogenic line across all isofemale lines to control for female × male × male interactions (Fricke *et al.*, 2010; Droge-Young *et al.*, 2012; Travers *et al.*, 2016). For the rival (first) matings, sexually naïve tester males were placed in individual vials with a single virgin tester female and observed. Following a similar protocol to the sperm offence assay carried out by Arbuthnott *et al.* (2014), the first male was removed from the vial after each mating was complete and replaced immediately with a focal male (from one of the isofemale lines). Pairs were observed continuously for 5 h. After a mating pair disengaged, the female was removed from the vial and placed into a fresh vial. Females were then transferred to new food vials every 24 h for 3 days. After eclosion, progeny were scored for eye colour. Tester females and focal isofemale line males were all recessive for red eyes, whereas rival tester males (P_1) were brown eye dominant. Therefore, the proportion of red-eyed progeny produced was taken as a measure of P_2. To control for instances of non-sperm representation that may have occurred due to failed inseminations (Garcia-Gonzalez, 2004), only females that produced both brown- and red-eyed offspring were included in the final analyses.

We measured egg-to-adult survival in offspring of isofemale line males to control for the confounding effect of differential egg-to-adult viability on P_2 estimates (Gilchrist & Partridge, 1997; Garcia-Gonzalez, 2008a). An average of 5.45 ± 1.47 males from each isofemale line were mated monogamously to a virgin tester female from the same isogenic line as the tester females used in the P_2 assay. Males and females were paired in individual yeasted vials for 48 h before the assay commenced to ensure that the pair was mated and the female ready to oviposit. To measure egg-to-adult viability of offspring from the focal males, each pair was transferred to a vial with blue food dye for 24 h to allow the female to oviposit. The eggs laid in this period were counted and the number of adults that emerged was used to calculate egg-to-adult viability.

Sex combs

We counted the number of teeth on the sex comb of the right leg for each of 4.8 ± 1.1 males per isofemale line. We carefully removed the foretarsus of the right front leg from the body and placed it onto double-sided transparent tape on a microscope slide. The number of teeth on the sex comb was counted under a compound microscope at 200× magnification against a white background.

Wing size

We measured male wing size as a proxy for body size for 4.5 ± 1.3 males per line. The right wing from each male assessed for sex comb size was removed using forceps, dipped in Histoclear (National Diagnostics,

Atlanta, GA, USA) and mounted under a cover slip on a glass slide using Aquamount (Thermo Scientific, Waltham, MA, USA). When the slides were set, wings were photographed using a digital camera attached to a compound microscope at 40× magnification. The photos were saved as TIFF images and analysed using the Object Image software (Vischer *et al.*, 1994). We placed landmarks on each wing to calculate the wing area as described in Gilchrist & Partridge (1999).

Cuticular hydrocarbons

To quantify differences in CHC profiles across isofemale lines, we sampled CHCs from 7.7 ± 2.0 four-day-old virgin males per line. Males were housed in individual fresh food vials from eclosion to prevent cross-contamination of CHCs between individuals (Everaerts *et al.*, 2010). Previous findings in this species suggest plastic changes in CHC profiles in response to social cues (Krupp *et al.*, 2008). To ensure that males expressed CHC profiles that are relevant for sexual signalling, we exposed the males to visual contact with females by placing securely closed male vials into a population cage containing 200 virgin females for 15 min. After the visual exposure to females, males were individually plunged into vials containing 30 μL hexane with 100 ng n-hexacosane as an internal standard for 5 min at room temperature. We injected 3 μL of each sample in to a 7890A gas chromatograph and 5975C mass spectrometer operating in splitless model, and fitted with an Agilent column of 20 m × 0.15 mm internal diameter. The column was held isothermally at 140 °C and then programmed at a rate of +3 °C min^{-1} to 300 °C. Helium was used as the carrier gas at a linear velocity of 43 cm s^{-1}. The injector port was set at 280 °C. The transfer line from the gas chromatograph to the mass spectrometer was set at 250 °C. We also analysed hexane blanks to control for potential contamination of samples. The compounds were identified in AMDIS and NIST 14, and by comparing their retention times with previously published *Drosophila* CHCs (Everaerts *et al.*, 2010). The compounds were quantified using the target ion in Mass Hunter Quantitative Analysis (version B.06.00; Agilent, Santa Clara, CA, USA).

Statistical analyses

CHC analysis

For data analysis, peaks were labelled by peak number, which corresponded to their retention times (see Table S1 and Fig. S1). We used proportional peaks that were calculated by dividing the area of each peak in a given sample by the sum of all peak areas in that sample (see Fig. S1 for chromatogram of compound peaks). To remove the problem of nonindependence introduced into the analysis using proportions, we used a log contrast transformation (Blows & Allan, 1998; Rundle *et al.*, 2005; Simmons *et al.*, 2014). Log contrasts were calculated by dividing each proportional peak by the internal standard peak area, and then taking the log $(x + 1)$ of the new variable, as described previously (Blows & Allan, 1998; Thomas & Simmons, 2009; Simmons *et al.*, 2014). We performed a principal components analysis (PCA) in (JMP®10, SAS, Cary, NC, USA) on compounds ($n = 15$) that could be identified in all samples.

Genetic variation in traits

We examined the differences in traits among isofemale lines in R version 3.1.2 (R Core Team, 2015) using analysis of variance (ANOVA) on untransformed wing area, number of sex comb teeth and male inhibition of female remating after all data and residuals were tested for normal distribution. We also analysed sex comb teeth using a quasi-Poisson generalized linear model (GLM) to correct for underdispersion and male inhibition of female remating using a Poisson GLM. However, we have not reported the findings from the quasi-Poisson or Poisson GLM as they do not differ from the ANOVAS. Linear model assumptions were violated for the CHC principal components (PCs). Thus, we analysed differences between isofemale lines for PCs using nonparametric tests (Kruskal–Wallis test). The lmer4 package (Bates *et al.*, 2015) was used to fit a linear mixed model to square-root-transformed courtship duration with the tester female line ID included as a fixed effect. Male identity was included as a random effect to account for repeated measures. Significance of the isofemale line as well as the tester female line was tested using likelihood ratio tests. Proportion data (male mating success, P_2 and egg-to-adult viability) were analysed on the logit scale (Eggert *et al.*, 2003; Engqvist, 2013) using GLMs fitted with a quasi-binomial distribution to account for overdispersion. We controlled for variation attributable to tester female mating partner by including female line ID as a fixed effect in our analysis of male mating success. Significance for differences between isofemale lines in mating success, P_2 and egg-to-adult viability was determined using likelihood ratio tests. To avoid type I errors associated with multiple testing, we adjusted *P* values for multiple testing using the Benjamini–Hochberg procedure implemented in R (Benjamini & Hochberg, 1995).

Relationship between male fitness and precopulatory traits

We ran three separate multiple regression analyses on mating success, inhibition of female remating and P_2 to investigate the relationship between measures of male fitness and precopulatory traits. Our response variables were mean trait values for males from each isofemale line. Line means for proportion data (mating success and P_2) were obtained by back-transforming coefficients from a binomial GLM (P_2) and a binomial GLMM

(mating success; with tester female line as a random effect). For the multiple regression analyses, linear models on logit-transformed mating success and P_2, and untransformed male inhibition of female remating line means were conducted with wing size, sex comb tooth number, male courtship duration and the four CHC principal components included as predictor variables in all three models. Egg-to-adult viability was an additional predictor variable included in the P_2 regression to account for among line variation in offspring viability (Gilchrist & Partridge, 1997). We obtained effect sizes, standard errors and *P* values from the full model to avoid biasing estimates through removal of nonsignificant terms (Forstmeier & Schielzeth, 2011).

The pairwise relationships between the three fitness components of male reproductive success were examined by Pearson correlations between untransformed remating inhibition and logit-transformed P_2 and mating success.

Results

Cuticular hydrocarbons

The PCA on the 15 peaks (Fig. S1) yielded four PCs with eigenvalues larger than one. The four PCs cumulatively explained 80.5% of the total variance in CHC composition. The first principal component (PC1) explained 54.8% of the variation, and was positively loaded to most peaks except for peak 2 and peaks 14–16 (Table S1). The second component (PC2) explained 11.4% and was loaded positively by peaks 14–16. PC1 and PC2 thus corresponded to the relative abundance of shorter and longer CHCs, respectively. PC3 explained 8.1%, and PC4 explained 6.8% of the variation. These PCs were both loaded positively by peak 2 but showed no clear pattern.

Genetic variation in traits

We found significant variation among isofemale lines in most of the traits measured (see Table 1 for trait test statistics and *P* values). Male mating success and P_2 showed highly significant differences among isofemale lines, whereas remating inhibition did not differ among lines. Of the precopulatory traits, wing size, sex comb tooth number and three of the four CHC principal components differed significantly among lines, whereas courtship duration and CHC PC1 did not meet significance criteria. Tester female line had a significant effect on male courtship duration ($F_{4,297} = 6.672$, $P < 0.001$) and a significant effect on male mating success ($\chi^2 = 39.476$, $P < 0.001$). The significant effect of female tester line encompasses variation in factors other than female genotype over the 5 days, such as male mating history. Thus, we refrain from further interpretation of this result. There was no correlation between isofemale line means for P_2 estimates and egg-to-adult viability ($z = 1.127$, $P = 0.633$). Variation in traits within and among isofemale lines is displayed in Fig. 1.

Relationship between male fitness and precopulatory traits

Figure 2 shows pairwise associations between isofemale line means for male traits and for our three components of male reproductive success. Multiple regression analyses on isofemale line means revealed no relationship between any of the traits examined and male mating success (Table 2; all $P > 0.3$). The analysis of remating inhibition revealed a negative association with courtship duration; males from isofemale lines that needed to court for shorter periods before mating inhibited female remating for longer (Table 2; $t = -2.51$, $P = 0.019$). However, a likelihood ratio test between the full model and a null model (including only the intercept) showed marginal support for any influence of male traits on remating inhibition (LRT: $\chi^2 = 19.88$, d.f. = 7, $P = 0.062$). Similarly, a comparison of the full model for P_2 to the null model showed that an influence of the phenotypic traits on P_2 was not supported overall (LRT: $\chi^2 = 18.89$, d.f. = 8, $P = 0.145$), despite the individual significant effect of courtship duration (Table 2; $t = -2.23$, $P = 0.036$).

There were no significant associations between the different episodes of sexual selection or the different components of male reproductive success (Fig. 3). Mean mating success (logit-transformed) did not correlate either with remating inhibition ($t = 0.77$, d.f. = 32, $P = 0.448$) or with P_2 (logit-transformed; $t = 0.86$, d.f. = 30, $P = 0.399$), and remating inhibition did not correlate with P_2 ($t = 1.11$, d.f. = 28, $P = 0.275$).

Discussion

Whereas the connection between episodes of sexual selection is receiving increased empirical attention, few studies have carried out comprehensive tests of the genetic relationships among traits determining pre- and post-copulatory reproductive success beyond the analysis of a limited number of isolated traits. Here, we inspect the associations, including the genetic basis underlying these associations, among a suite of morphological, chemical and behavioural traits, and their relationship with three components of fitness (mating success, remating inhibition and offensive sperm competitiveness). We found little evidence for covariation between male precopulatory traits and reproductive success. Importantly, we found no relationship between our different measures of reproductive success, which suggests that pre- and post-copulatory episodes of sexual selection act independently, at least with regard to the traits measured and the population of

Table 1 Trait variation between isofemale lines. Traits were measured for 34–40 isofemale lines derived from wild-caught females. The test statistic was chosen based on the distribution of the response variable and on whether normality assumptions for linear models were met. We adjusted *P* values using the Benjamini–Hochberg procedure to account for multiple testing.

Trait	*N* isofemale lines	*N* males/line (mean ± SD)	Test statistic	Test value	*P*-value	Adjusted *P*-value*
Male mating success	39	6.4 ± 1.0	Likelihood ratio test	86.896	< 0.001	**< 0.001**
P_2	34	2.8 ± 1.7	Likelihood ratio test	−364.82	< 0.001	**< 0.001**
Male courtship duration	39	4.3 ± 1.4	Likelihood ratio test	52.038	0.064	0.080
Egg-to-adult viability	39	5.45 ± 1.5	Likelihood ratio test	−436.2	< 0.001	**< 0.001**
Male remate inhibition	39	2.9 ± 1.3	ANOVA	0.686	0.898	0.898
Wing size	38	4.5 ± 1.3	ANOVA	6.005	< 0.001	**< 0.001**
Sex comb bristle number	38	4.8 ± 1.1	ANOVA	3.686	< 0.001	**< 0.001**
CHC PC1	40	7.7 ± 2.0	Kruskal–Wallis	52.588	0.072	0.090
CHC PC2	40	7.7 ± 2.0	Kruskal–Wallis	110.346	< 0.001	**< 0.001**
CHC PC3	40	7.7 ± 2.0	Kruskal–Wallis	96.437	< 0.001	**< 0.001**
CHC PC4	40	7.7 ± 2.0	Kruskal–Wallis	165.9	< 0.001	**< 0.001**

CHC, cuticular hydrocarbon.
Significant *P* values are highlighted in bold.
*Adjusted for multiple comparisons following Benjamini & Hochberg (1995).

D. melanogaster studied here. Our results, however, support previous evidence for the presence of genetic variation in male mating success, P_2, sex comb tooth number, body size and CHC composition.

Genetic variation in male fitness components

Our finding of significant variation among isofemale lines in male mating success is congruent with previous evidence for genetic variation in male attractiveness in *Drosophila* (Kosuda, 1985; Partridge *et al.*, 1985; Taylor *et al.*, 2007; Hosken *et al.*, 2008; Ingleby *et al.*, 2013b), other insect species (Moore, 1990; Wedell & Tregenza, 1999) and vertebrates (Siegel, 1972; Brooks & Endler, 2001; Evans, 2010). Quantitative genetic analyses have previously reported significant additive genetic variation in mating success in *D. melanogaster* (Hughes, 1995), among hemiclones derived from a large outbred population (Friberg *et al.*, 2005), and among recombinant inbred lines (Hughes & Leips, 2006). Whereas we found no variation among our isofemale lines in courtship duration, previous studies of *D. melanogaster* have provided evidence for heritable variation in male mating behaviours (Moehring & Mackay, 2004; Mackay *et al.*, 2005; Ruedi & Hughes, 2008; Gaertner *et al.*, 2015; but see Taylor *et al.*, 2013). In our study, we obtained measures of courtship duration in the males that successfully copulated with a female during a 20-min period. Therefore, it is possible that our data for this trait may only include the most attractive males that could gain matings in the limited time period, and hence may not represent the overall variation for courtship duration.

We found significant variation among isofemale lines in offensive sperm competitiveness (P_2). Previous findings for heritable variation in P_2 are mixed. Friberg *et al.* (2005) found low levels of additive genetic variation among hemiclonal lines of *D. melanogaster* and Nandy *et al.* (2013) found a decrease in offensive sperm competitiveness under relaxed selection in lines with a female-biased sex ratio. Other studies have found a number of polymorphisms that contribute to sperm competitiveness in *D. melanogaster* (Fiumera *et al.*, 2007; Zhang *et al.*, 2013; Reinhart *et al.*, 2015). However, Arbuthnott *et al.* (2014) found no evidence for variation in P_2 among eight replicate populations of *D. melanogaster*, a quantitative genetic study also failed to detect significant additive genetic variation in P_2 (Hughes, 1997), and an experimental evolution study failed to generate divergence in either P_1 or P_2 (Bjork *et al.*, 2007). Findings from other species also provide conflicting evidence, with support for additive genetic variance in sperm competitive ability in some (Radwan, 1998; Hosken *et al.*, 2001; Konior *et al.*, 2005; Simmons & Garcia-Gonzalez, 2008) but not in other species (for a review, see Simmons & Moore, 2009). The presence of additive genetic variation in sperm competitiveness is a prerequisite for the sexually selected sperm process, which proposes that post-copulatory female choice for sperm competitiveness could contribute to the evolution of polyandry (Keller & Reeve, 1995; Evans & Simmons, 2008). Our finding of significant variance among isofemale lines in this trait may have been facilitated by controlling for male × male × female interactions through the use of rival males and females from isogenic lines. However, our recent quantitative genetic study of the assumptions of the sexually selected sperm hypotheses in which we also used isogenic nonfocal individuals in a different population of *D. melanogaster* from Queensland, Australia, found low and nonsignificant levels of additive genetic variation in P_2 (Travers *et al.*, 2016). The reason for genetic variation in P_2 within some populations and species but not others is thus unresolved. Differing selection pressures on the trait between populations or bottlenecks and founder

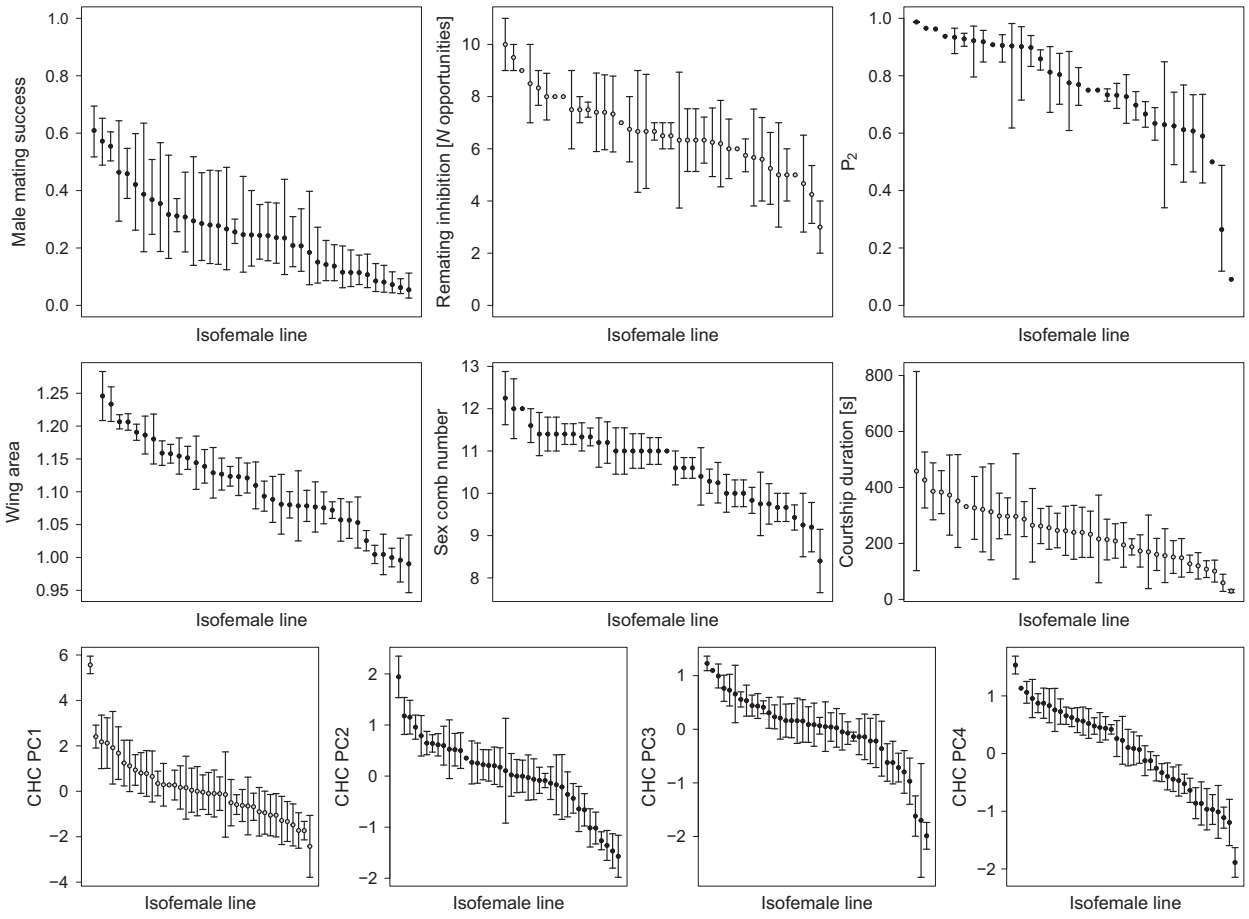

Fig. 1 Male trait variation within and between isofemale lines. Points and error bars indicate isofemale line means and standard errors. Black points are given for traits with significant differences between isofemale lines, and grey points indicate traits that were not significantly different between lines (see main text and Table 1). Isofemale lines were sorted by decreasing mean values.

effects could influence the amount of additive genetic variation available to respond to selection in different populations. Likewise, selective sweeps may regularly erode genetic variation and whether this variation is detected may depend on how recently the last sweep occurred.

An important component of a male's defensive strategy to prevent sperm competition with other males is his ability to suppress female receptivity to remating (Chapman, 2001). A single accessory gland peptide, the sex peptide, has been identified as playing a major role in prolonging the female refractory period (Chen *et al.*, 1988; Aigaki *et al.*, 1991; Kalb *et al.*, 1993; Chapman, 2001). Experimental evolution studies in *Drosophila* have found strong evidence for divergence in male ability to inhibit remating in females (Rice, 1996; Holland & Rice, 1999; Pitnick *et al.*, 2001b), suggesting that the trait must have significant levels of additive genetic variance. Here, however, we found no variance among isofemale lines in this trait. Another study that investigated genetic variation in male-induced refractoriness in a natural population of *D. melanogaster* similarly failed to detect significant genetic variation (Fiumera *et al.*, 2007). The lack of significant variation in our study may be due to a history of strong directional selection on remating inhibiting seminal fluid proteins that may have resulted in the erosion of genetic variance in this trait.

Genetic variation in traits thought to be subject to sexual selection

The significant variation across isofemale lines in most traits indicates that there is sufficient genetic variation in precopulatory traits within this population to respond to sexual selection.

Sex combs are used by males to grasp the female abdomen and to spread her wings during copulation (Spieth, 1952). QTL studies suggest additive genetic variation that significantly affects sex comb tooth number in *D. melanogaster* (Nuzhdin & Reiwitch, 2000; Kopp *et al.*, 2003) and *D. simulans* (Tatsuta & Takano-Shimizu,

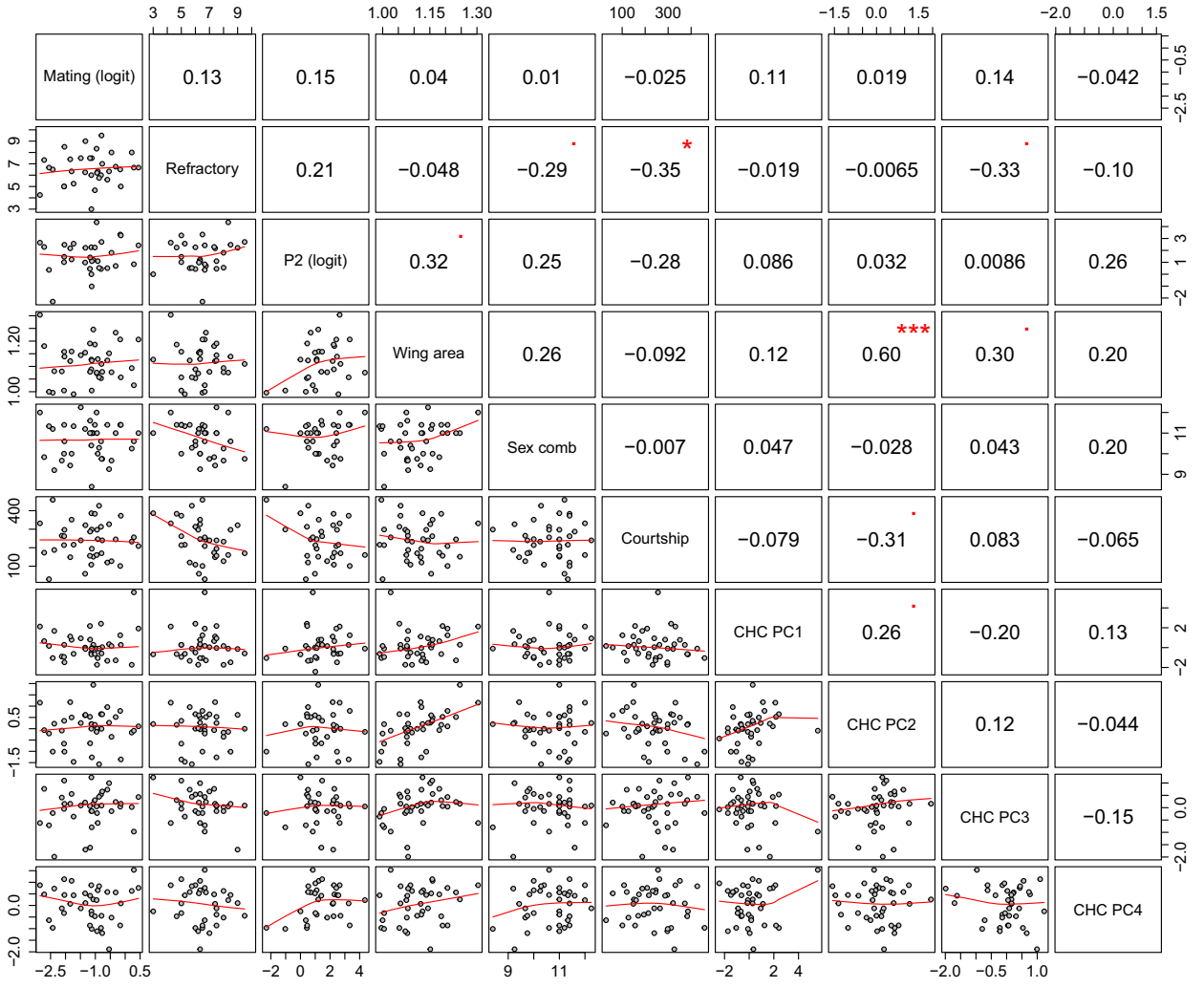

Fig. 2 Correlation matrix of precopulatory traits and fitness traits. The lower panel shows the pairwise associations between the isoline means [mating success (logit-transformed), remating inhibition and P_2 (logit-transformed), body size, sex comb number, courtship duration, CHC PC1, CHC PC2, CHC PC3 and CHC PC4]. The upper panel shows Pearson's rho for all pairwise correlations, and significance (P values not adjusted for multiple testing) is indicated by the red symbols (***$P < 0.001$, *$P < 0.05$, °$P < 0.1$). There was very limited support for correlations between phenotypic trait means and male fitness components (see main text and Table 2). CHC, cuticular hydrocarbon.

2006). In agreement with our findings in *D. melanogaster*, significant variance among isofemale lines has also been reported for *D. simulans* and *Drosophila sechellia* (Macdonald & Goldstein, 1999). Within-population genetic variance is also supported by divergence through artificial selection for tooth comb number in *D. melanogaster* (Ahuja & Singh, 2008). Thus, in principle sexual selection could affect evolutionary change in sex comb morphology. As for body size, this trait is known to be influenced in *Drosophila* by a number of environmental factors such as temperature and diet (Hillesheim & Stearns, 1991; Partridge *et al.*, 1994; De Moed *et al.*, 1997; Norry & Loeschcke, 2002), but our finding of significant variation between lines in wing area is consistent with previous findings for genetic variation in body size (Coyne & Beecham, 1987; Cowley & Atchley, 1988; Ruiz *et al.*, 1991; Partridge & Fowler, 1993; Partridge *et al.*, 1994). We also looked at variation in CHCs, which act as sex pheromones during courtship. We found significant variation among isofemale lines in the second, third and fourth principal components, which collectively explained 26% of the variation in CHC composition. Variance in the first principal component, which explained 54% of the variation, did not reach statistical significance. Scott *et al.* (2011) also reported significant variation in CHC profiles across four isofemale lines of *D. melanogaster*. Studies of *Drosophila* generally (Ferveur & Jallon,

Table 2 Linear model summaries for full models on our three components of male reproductive success. Predictor and response variables were isofemale line means. Proportion response variables (mating success and P_2) were logit-transformed to meet normality assumptions. Full models comprised all recorded predictor variables without interaction terms. Full models were compared to null (intercept-only) models using likelihood ratio tests.

Response variable	Isofemale lines	Fixed effects	Estimate	SE	*t*-value	*P*
Mating success	*N* = 38	Intercept	−1.277	3.200	−0.400	0.692
(logit-transformed)		Wing area	0.222	2.900	0.077	0.939
		Sex comb number	−0.005	0.182	−0.027	0.979
		Courtship duration (s)	−0.001	0.002	−0.377	0.709
		CHC PC1	0.114	0.117	0.976	0.337
		CHC PC2	−0.111	0.284	−0.391	0.699
Comparison to null model:		CHC PC3	0.260	0.257	1.012	0.320
$\chi^2 = 1.39$, $P = 0.975$		CHC PC4	−0.060	0.208	−0.289	0.774
Remate inhibition	*N* = 33	Intercept	9.056	4.816	1.881	0.072
		Wing area	4.492	3.952	1.137	0.267
		Sex comb number	−0.574	0.291	−1.971	0.060
		Courtship duration	**−0.006**	**0.002**	**−2.514**	**0.019**
		CHC PC1	0.028	0.161	0.176	0.862
		CHC PC2	−0.560	0.420	−1.332	0.195
Comparison to null model:		CHC PC3	−0.466	0.498	−0.937	0.358
$\chi^2 = 19.88$, $P = 0.062$		CHC PC4	−0.265	0.283	−0.935	0.359
P_2 (logit-transformed)	*N* = 31	Intercept	−8.242	4.764	−1.730	0.098
		Egg-to-adult viability	0.313	1.766	0.178	0.861
		Wing area	7.894	4.172	1.892	0.072
		Sex comb number	0.171	0.300	0.569	0.575
		Courtship duration	**−0.005**	**0.002**	**−2.232**	**0.036**
		CHC PC1	0.117	0.179	0.656	0.519
		CHC PC2	−0.767	0.409	−1.877	0.074
Comparison to null model:		CHC PC3	0.165	0.441	0.373	0.713
$\chi^2 = 18.89$, $P = 0.145$		CHC PC4	0.265	0.355	0.745	0.464

CHC, cuticular hydrocarbon.
Predictor variables with a significant effect are highlighted in bold.

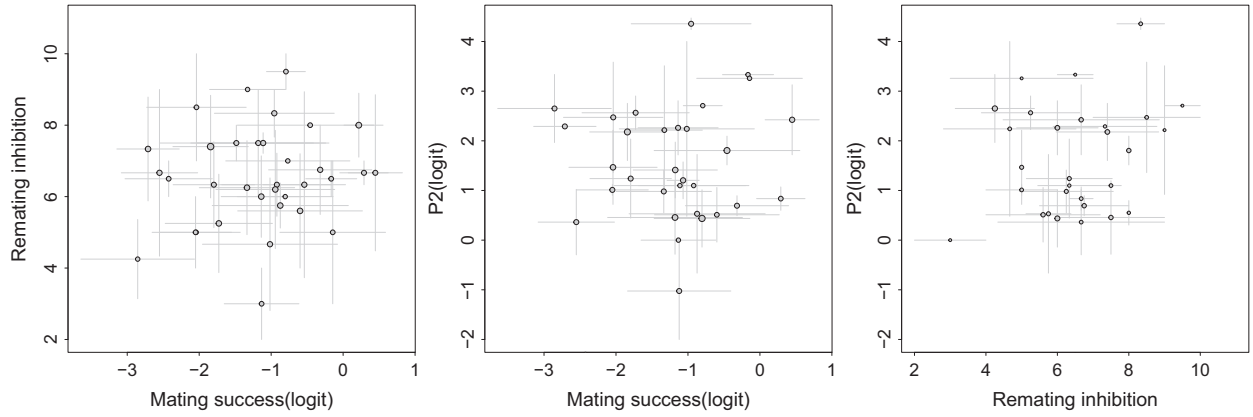

Fig. 3 No significant correlations between the three fitness indices. Shown are the pairwise associations between mating success (logit-transformed), remating inhibition and P_2 (logit-transformed). Points and error bars indicate isofemale line means and standard errors. The point surface areas correspond to variation in the mean number of individual males assessed for the two traits of interest for a given isofemale line. None of the correlations were significantly different from zero (see main text).

1996; Ferveur, 2005; Sharma *et al.*, 2012; Weddle *et al.*, 2012), and other insect taxa (Thomas & Simmons, 2009), have reported genetic variance in CHC profiles. In *D. melanogaster*, Foley *et al.* (2007) found a number of QTL associated with differences in CHC profile in a natural population. Overall, our findings support significant genetic variation in CHC composition in this population of *D. melanogaster*, and thus suggest that CHCs are capable of responding to sexual selection.

Relationship between male fitness and precopulatory traits

Our multiple regression analyses revealed a negative association between courtship duration and post-copulatory success: isofemale lines with shorter courtship duration (more attractive males) inhibited female remating for longer and were more successful in offensive sperm competition, which may reflect an energetic trade-off between the two traits. Whereas our models obtained only limited statistical support, these findings are similar to a significant positive genetic correlation between copulation speed and fertilization success reported for *D. simulans* (Hosken *et al.*, 2008). However, other traits measured, such as body size and sex comb size, which have previously been associated with precopulatory mating success and/or sperm competitiveness (e.g. Partridge & Farquhar, 1983; Partridge *et al.*, 1987a,b; Rybak *et al.*, 2002; Polak & Simmons, 2009; Scott *et al.*, 2011) showed no genetic correlation with our three measures of male fitness or with each other.

Theory predicts antagonistic selection between defensive and offensive adaptations to sperm competition (Parker, 1970a,b). Numerous studies have therefore investigated the relationship between males' sperm defence ability and ability to displace previously stored sperm (sperm offence) (Clark *et al.*, 1995; Civetta & Clark, 2000; Nilsson *et al.*, 2003; House & Simmons, 2006; Fricke *et al.*, 2010). Here, we found no relationship between male remating inhibition ability and offensive sperm competitiveness (P_2). This is in agreement with Fiumera *et al.* (2007) who found no relationship between refractory induction and P_2 across chromosome substitution lines of *D. melanogaster*. Other studies on *D. melanogaster* and *Tribolium castaneum* support this lack of a genetic association between defensive and offensive sperm competitiveness (Clark *et al.*, 1995; Civetta & Clark, 2000; Nilsson *et al.*, 2003). In contrast, Friberg *et al.* (2005) found a significant positive relationship between P_2 and remating inhibition among hemiclones from a natural population of *D. melanogaster*. The lack of a relationship between males' ability to inhibit female remating and P_2 in the population of flies that we have studied suggests limited overlap of genes contributing to each trait. Hence, they may both be able to evolve independently without constraints imposed by their underlying genetic architecture.

We found no relationship between sex comb tooth number and male fitness. Evidence that sex combs are under sexual selection has so far been mixed. Both surgical and genetic ablation experiments showed that the absence of sex combs results in higher rejection from females and reduced mating success (Spieth, 1952; Cook, 1977; Ng & Kopp, 2008). In *Drosophila bipectinata*, studies of a wild population found that males caught copulating with females had significantly more sex comb teeth than noncopulating males, suggesting sexual selection for increased sex comb size (Polak *et al.*, 2004). Likewise, a study of *D. simulans* found copulating males had fewer sex comb teeth than noncopulating ones, whereas for *Drosophila pseudoobscura* sex comb size did not differ significantly between copulating and noncopulating males (Markow *et al.*, 1996). In a field-based study on the same natural population of *D. melanogaster* from which the isofemale lines were drawn for this study, Robinson *et al.* (2012) found no evidence of sexual selection acting on either body size or sex comb tooth number. Moreover, in their experimentally evolving lines, Snook *et al.* (2013) found no effect of enforced monogamy or increased levels of sexual selection on tooth comb number for either *D. melanogaster* or *D. pseudoobscura*. Whereas most studies of sex comb tooth number have investigated the relationship between tooth number and mating success, Polak & Simmons (2009) investigated its relationship with P_2 in *D. bipectinata*, finding a positive relationship. Collectively, these findings indicate that whereas there may be substantial variation for sex comb tooth number, there remains little clear evidence that the sex comb tooth number is subject to pre- and/or post-copulatory sexual selection.

Larger males usually have an advantage in sexual selection for mates via female choice and intrasexual competition (Partridge *et al.*, 1987a; Andersson, 1994). We found no relationship between isofemale line mean body size and precopulatory male success in our non-competitive mating trials. This was in contrast with a number of studies in this species that have found a mating advantage among larger males compared to smaller males in non-competitive mating arenas due to more vigorous courtship (Partridge *et al.*, 1987b), and more intense courtship song (Partridge *et al.*, 1987a) which may contribute to the faster mating speed of larger males (Partridge & Farquhar, 1983; Jagadeeshan *et al.*, 2015). However, our finding is consistent with Robinson *et al.*'s (2012) field-based study of the population from which our flies were drawn; in the field larger males did not appear to have a mating advantage. Nevertheless, larger males have been reported to have a mating advantage due to greater territorial success and their ability to dominate smaller males (Ewing, 1964; Partridge & Farquhar, 1983; Partridge *et al.*, 1987b).

The relationship between post-copulatory success and body size is unclear. One hypothesis posits that larger males will have higher post-copulatory success because they can produce larger quantities of sperm and/or seminal fluid proteins. Specifically, seminal fluid proteins produced by the accessory glands strongly influence male post-copulatory success (Wolfner, 1997), and evidence indicates that greater amounts result in higher success (Kalb *et al.*, 1993). Bangham *et al.* (2002) thus investigated the effect of body size, accessory gland size and testis size on male mating success and found a positive phenotypic relationship between accessory gland size and mating success, and that larger males had

higher post-copulatory success than smaller males, suggesting that precopulatory selection on body size is reinforced in the post-copulatory episode of sexual selection. However, it is also recognized that post-copulatory sexual selection can have antagonistic effects on male size (Danielsson, 2001). In two different populations of *D. melanogaster*, a negative relationship between male size and female longevity has been found (Pitnick & Garcia Gonzalez, 2002; Friberg & Arnqvist, 2003). Therefore, whereas selection may act on male body size through female mate choice, the cost to female fitness of mating with preferred males could result in stabilizing selection on male size. However, for the population of flies used in our study we found no evidence of a relationship between male size and post-copulatory reproductive success. Our finding of no genetic relationship between body size and reproductive success in either the pre- or post-copulatory stage highlights that patterns of genetic covariances among traits are not necessarily reflected by phenotypic correlations.

In *Drosophila*, CHCs can differ between the sexes, and have been reported to be involved with mate recognition and mate choice (Ferveur, 2005). For example, in *Drosophila serrata*, CHCs are sexually selected as a consequence of female choice in both a laboratory population (Blows *et al.*, 2004) and wild-caught flies (Hine *et al.*, 2004; Petfield *et al.*, 2005). The compound 7-tricosene has been shown previously to be preferred by females in *D. melanogaster* (Grillet *et al.*, 2006), but we found no relationship between any of our three fitness measures and PC1, which was the PC on which 7-tricosane had the strongest loading. However, consistent with findings from our population, Scott *et al.* (2011) found significant variation among isofemale lines of *D. melanogaster* in both mating success and CHCs, but variation in CHCs was not associated with mating success. Likewise, in their study of *D. simulans* Ingleby *et al.* (2013a) found genotype-by-environment interactions in CHC composition across different dietary and temperature environments but no change in male attractiveness, suggesting that CHCs may not be reliable sexual signals across environments. We found no relationship between any of the four CHC principle components and male fitness. Thus, the role of CHCs in precopulatory sexual selection may also vary widely across *Drosophila* species.

We maintained the isofemale lines for 10 generations in the laboratory before we measured the traits. Thus, genetic drift due to inbreeding may have resulted in unpredictable changes in allele frequencies within the lines (Lande, 1980). It has been shown that genetic drift can change both the magnitude and orientation of covariances among traits (Phillips *et al.*, 2001). In a comparison of G matrices from 52 inbred lines of *D. melanogaster* generated from an outbred population, Phillips *et al.* (2001) showed that whereas the structure of the *G* matrix averaged over all inbred lines was similar to that of the outbred control population, individual inbred lines showed considerable variation in the orientation (sign) and magnitude of the genetic variance and covariance. Consequently, we cannot rule out a potential influence of drift on the genetic covariances.

Interactions between pre- and post-copulatory selection

We found little evidence for any significant covariation between episodes of pre- and post-copulatory sexual selection in this population of flies. Previous studies of *D. melanogaster* that have investigated the interplay between pre- and post-copulatory episodes of selection have returned similar findings. Using rival males from a transgenic line that expressed a green fluorescent protein marker, Droge-Young *et al.* (2012) conducted competitive mating trials to determine variation in reproductive success attributable to pre- and post-copulatory episodes of sexual selection, and their associations with offspring viability. They measured male attractiveness via mating latency and correlated offspring viability with traits relevant to precopulatory sexual selection, such as thorax length, and traits relevant to post-copulatory success, such as copulation duration, P_1, P_2, and male ability to induce refractoriness. They found no relationship between sperm competitive success assessed from paternity measured in eggs and offspring viability, and no correlation between pre- and post-copulatory sexual selection (Droge-Young *et al.*, 2012). Whereas we found substantial variation between isofemale lines in egg-to-adult viability, indicating a heritable component to the trait, our regression analysis on line means showed no relationship between egg-to-adult viability and P_2 estimates. This also suggests that our finding of genetic variation in P_2 was not driven by differential egg-to-adult viability, which if not accounted for can confound estimates of fertilization success (Gilchrist & Partridge, 1997; Garcia-Gonzalez, 2008b; Droge-Young *et al.*, 2012).

Studies of other species have found genetic covariance between pre- and post-copulatory sexually selected traits. For example, Evans (2010) found a quantitative genetic trade-off between precopulatory attractiveness and ejaculate quality in guppies. In the scorpion fly *P. cognata*, Engqvist (2011) found a significant negative genetic association between attractiveness and nuptial salivary secretions, a trait previously shown to be related to sperm transfer and hence fertilization success in this species (Engqvist & Sauer, 2003). The direction of the correlation between pre- and post-copulatory traits can even change depending on resource availability (Mehlis *et al.*, 2015). Studies of wild populations, where resource availability may strongly differ between individual males, have revealed positive genetic correlations between sexual display and ejaculate quality (Chargé *et al.*, 2012). Although trade-offs between pre- and post-copulatory sexual traits offer

a solution to the maintenance of genetic variation in fitness traits, empirical evidence remains limited (see, for instance, Mautz *et al*., 2013). We found no relationship between episodes of sexual selection on male *D. melanogaster*, which suggests that pre- and post-copulatory sexual selection operate separately, at least in the population studied, so that pre- and post-copulatory traits may be relatively free to evolve independently.

Conclusion

Whereas phenotypic studies investigating the integration of pre- and post-copulatory selection are accumulating, our study provides one of only a few studies to have investigated the underlying genetic basis and genetic relationships between pre- and post-copulatory episodes of sexual selection. The significant variation between isofemale lines suggests the presence of genetic variance in precopulatory male traits and fertilization success. However, the weak evidence for genetic covariance between the pre- and post-copulatory traits found in this study suggests that selection may act independently on individual episodes. Hence, males may be able to optimize their fitness through these routes without genetic constraints.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1 Principal components analysis of relative CHC peak abundance.

Figure S1 GC-MS chromatogram after full body extraction in hexane.

Data deposited at Dryad: doi: 10.5061/dryad.fg7jv

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