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Masculinization of gene expression is associated with male quality in *Drosophila melanogaster*

Rebecca Dean,[1,2,3] Camille Hammer,[1] Vanessa Higham,[1] and Damian K. Dowling[1]

[1]*School of Biological Sciences, Monash University, Clayton, Victoria 3800, Australia*

[2]*Department of Genetics, Evolution and Environment, University College London WC1E 6BT, United Kingdom*

[3]*E-mail: R.dean@ucl.ac.uk*

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The signature of sexual selection has been revealed through the study of differences in patterns of genome-wide gene expression, both between the sexes and between alternative reproductive morphs within a single sex. What remains unclear, however, is whether differences in gene expression patterns between individuals of a given sex consistently map to variation in individual quality. Such a pattern, particularly if found in males, would provide unambiguous evidence that the phenotypic response to sexual selection is shaped through sex-specific alterations to the transcriptome. To redress this knowledge gap, we explored whether patterns of sex-biased gene expression are associated with variation in male reproductive quality in *Drosophila melanogaster*. We measured two male reproductive phenotypes, and their association with sex-biased gene expression, across a selection of inbred lines from the Drosophila Genetic Reference Panel. Genotypes with higher expression of male-biased genes produced males exhibiting shorter latencies to copulation, and higher capacity to inseminate females. Conversely, female-biased genes tended to show negative associations with these male reproductive traits across genotypes. We uncovered similar patterns, by reanalyzing a published dataset from a second *D. melanogaster* population. Our results reveal the footprint of sexual selection in masculinising the male transcriptome.

KEY WORDS: **DGRP, gene expression, sex-biased genes, sexual selection, sexual conflict.**

Sex-specific regulation of gene expression is thought to facilitate the evolution of phenotypic sexual dimorphism from a genome that is largely shared by both sexes (Parisi et al. 2004; Kopp et al. 2008; Mank 2009; Parsch and Ellegren 2013; Dean and Mank 2016). As a result, thousands of genes show sex-biased expression across numerous taxa (Ranz et al. 2003; Yang et al. 2006; Ellegren and Parsch 2007; Reinius et al. 2008). Male-biased genes (those with higher expression in males compared to females) are thought to typically encode male functions (Mank 2009), and they tend to have higher rates of evolution (Ranz et al. 2003; Harrison et al. 2015), potentially as a result of more intense sexual selection acting on males (Andersson 1994). Conversely, female-biased genes (those with higher expression in females compared to males) are thought to encode female functions. Sex-biases in gene expression therefore offer a key link in understanding how sex-specific selection acting on the phenotype shapes the evolution of the genome (Mank et al. 2013). To date, this relationship has principally been explored at two phenotypic scales; between the sexes (Hollis et al. 2014; Immonen et al. 2014; Harrison et al. 2015), and between alternative morphs within a single sex (Snell-Rood et al. 2011; Pointer et al. 2013; Stuglik et al. 2014; Dean et al. 2017). However, one phenotypic scale that has not yet been addressed is whether patterns of sex-biased gene expression reflect within-sex variation in individual quality.

A growing body of evidence suggests that sexual selection drives the evolution of sex-biased gene expression. For example, studies that applied divergent levels of sexual selection on

replicate populations of *Drosophila* have found that reducing the intensity of sexual selection led to the evolution of feminized gene expression (i.e., an increase in expression of female-biased genes) in both females and males (Hollis et al. 2014; Immonen et al. 2014). Across longer evolutionary timescales, species experiencing more intense sexual selection, as reflected in both their degree of sexual ornamentation and indices of sperm competition, have a higher proportion of genes with male-biased expression than species experiencing less intense sexual selection (Harrison et al. 2015).

Sex-biased gene expression has also been shown to facilitate the evolution of alternative mating tactics within a single sex (Snell-Rood et al. 2011; Pointer et al. 2013; Stuglik et al. 2014; Dean et al. 2017). For example, in the wild turkey (*Meleagris gallopavo*), the degree of elaboration of male secondary sexual characteristics scales with sexual dimorphism in gene expression (Pointer et al. 2013). Male turkeys can either become dominant or subordinate reproductive morphs (Krakauer 2008). Dominant male morphs have more elaborate, sexually selected plumage ornamentation and exhibit higher levels of expression of male-biased genes (i.e., more masculinized) compared to subordinate males that have less elaborate ornamentation (Pointer et al. 2013). These dominant male morphs also exhibit lower expression of female-biased genes (i.e., defeminized expression), compared to subordinate morphs. However, a somewhat contrasting pattern was observed in the ocellated wrasse (*Symphodus ocellatus)*, a species that also exhibits alternative male morphs, but with morphs that differ in both the level of sexual ornamentation (Alonzo 2008; Alonzo and Heckman 2010) as well as the level of sperm competition intensity experienced (Alonzo and Warner 2000). In ocellated wrasse, there are three male morphs. Territorial nesting males are brightly colored and are preferred by females, satellite males associate with a nesting male, and sneaker males are the smallest male morph that attempt to procure fertilizations through subterfuge. Sneaker males are the lowest quality male morph with the lowest reproductive success (Alonzo et al. 2000), and have low expression of both male- and female-biased gonadal genes. Satellite males experience a higher intensity of sperm competition than territorial males and have more masculinized (and defeminized) gene expression in the gonad than the territorial males (Dean et al. 2017). However, contrary to the patterns seen in the turkey, the most ornamented, territorial morph does not express the most masculinized gene expression profile. Thus, these two studies combined suggest that sexual selection shaped transcriptomic signatures of precopulatory selection in the turkey, and postcopulatory selection in the wrasse. Taken together, these results suggest that sexual selection indeed has the capacity to masculinize (and defeminize) patterns of gene expression throughout the transcriptome. However, whether these patterns of masculinization of gene expression extend to species without distinct male morphs apparent to human eyes, remains to be tested.

Under the assumption that male-biased expression confers phenotypic effects of male-benefit and female-detriment, while female-biased expression confers the converse (Mank 2009), we may predict that variation in expression levels of male-biased genes will lie at the heart of population-level variation in male quality. Expression levels of certain male-biased genes, with known effects on components of male reproductive fitness, are likely to contribute to variation in male quality within a population. For example, many genes on the mammalian Y chromosome play a major role in male fertility (Lahn and Page 1997). However, many sexually selected traits are likely to be polygenic in their underlying genetics (Gleason et al. 2002; Chenoweth et al. 2008; Poissant et al. 2008), as demonstrated by sexually selected traits associating with many quantitative trait loci with only small effect (Limousin et al. 2012; Randall et al. 2013; Veltsos et al. 2015). This has also recently been illustrated in a study on sperm morphology and swimming speed, polygenic traits that predict fertilizing advantage in zebra finch (Kim et al. 2017). In particular, 108 genes were differentially expressed between lines of zebra finch that were under artificial selection for long- and short-sperm length (Kim et al. 2017). These genes were overrepresented on the avian Z chromosome and tended to be upregulated in long-sperm lines (Kim et al. 2017). Just as upregulation of many genes contributes to variation in these sperm traits in the zebra finch, higher expression of an aggregate of male-biased genes (and lower expression of female-biased genes), each with small effect, may be important in determining variation in male quality in general.

In this study, therefore, we aimed to test whether variation in male quality is positively associated with the expression of male-biased genes, and negatively associated with the expression of female-biased genes, in *D. melanogaster*. We used the Drosophila Genetic Reference Panel (DGRP) (Mackay et al. 2012; Huang et al. 2014), which consists of inbred lines derived from a population collected in Raleigh (North Carolina, USA). We measured two different aspects of male mating behavior; latency to copulate and number of females inseminated within a defined period of time. We first tested whether these two aspects of male quality were correlated with gene expression. We next tested whether the strength and direction of the associations between phenotype and gene expression were affected by sex-biased expression of the gene (i.e., whether the gene was male- or female-biased). We also performed a Genome Wide Association (GWA) to locate SNPs that associate with these phenotypes. Finally, we compared our results to those of a different population, by analysing patterns of gene expression and male fitness under competitive conditions in the LH_M laboratory population (a wild-type outbred population) of *D. melanogaster*, using the dataset of Innocenti and Morrow (2010).

Methods

FLY CULTURING

Flies were maintained on a cornmeal-molasses-agar diet (Ayroles et al. 2009), under a 12:12 light:dark cycle at 25°C. We used 33 (out of the core 38) DGRP lines, which were available in the laboratory of the authors. Each DGRP line was propagated by culturing eight males and eight females per 40 mL vial (each vial containing 6 mL of food medium), with three vials per DGRP line. These vials were propagated by culling the number of eggs per vial to 100, with flies transferred to fresh vials across three successive days (i.e., a total of nine vials per DGRP line).

GENERATING A "RALEIGH MIXED" POPULATION

We created an outbred population of flies, which was used to source females (hereafter "tester" females) that would be mated to the focal DGRP-line males in the experiments measuring male quality. To this end, five virgin males and five virgin females were collected from each of the 33 DGRP lines, and all the individuals combined and cultured in a 250 mL bottle containing 60 mL food medium. After the first generation, we maintained this population across 50 vials, each propagated by eight pairs, and standardising egg density to 100 eggs per vial. Adult offspring to emerge from these 50 vials were then admixed during culturing each generation before being redistributed back out to 50 fresh vials. The Raleigh-mixed population was maintained in this way for three generations before the experiment started. Virgin females from this population were collected, and stored in groups of five per vial, for use as tester females in the experiments described below. Vials containing these tester females were checked prior to the experimental assays of male quality to ensure the absence of larval activity within the vials (thus guaranteeing the females were all virgins).

MEASURING MALE QUALITY

Male mating behaviors were tested against four-day old virgin tester females from the Raleigh-mixed population. The diets of these tester females were supplemented with two doses of a standardized yeast solution (20 μL of 0.16 g/mL yeast slurry solution, per group of females, made from reverse osmosis water), the first provided 2.5 days prior to the behavioral assays, and the other provided immediately prior to the assays.

A single four-day old male from each DGRP line was transferred, by aspiration, into a vial of five virgin females. Observations were made of time taken for the male to initiate copulation. If males failed to mate within 120 minutes following their introduction to the tester-female vial, a maximum value for latency to copulate of 120 minutes was assigned to that male. Assays started 3 hours after the lights came on in the temperature-controlled room in which the flies were maintained, to coincide with peak mating activity (Sakai and Ishida 2001). The DGRP lines were tested in a randomised order. Observations were carried out in a temperature-controlled laboratory set to 25°C. Two replicates (flies) per DGRP line were tested per experimental sampling block, for a total of six blocks, where each block was a separate generation of flies. Once the latency to copulate assays were completed, each vial–containing the five tester females and one focal male–was placed back in the incubator, to cohabit for 24 h.

Following this first 24 h period of cohabitation, each focal male was transferred to a fresh vial containing another five virgin 4-day-old tester females that had been given the standardized yeast supplements (20 μL of solution 2.5 days and immediately prior to the introduction to the DGRP male), and provided with another 24 h period of cohabitation with this new set of females. The five females from the first cohabitation period were each transferred to their own individual vial, thus kept in singleton, each vial of which had 5 μL of yeast solution added to the surface of the food medium. A small incision was made in the surface of the food medium to facilitate normal levels of fecundity when females lay in isolation (Rice et al. 2005; Long and Rice 2007). After the second 24 h period of cohabitation, each focal male was discarded, and the second set of five tester females were also each transferred to their own yeasted-supplemented vial, with the food once again cut with a spatula to encourage egg laying.

Following cohabitation, the tester females were provided with 24 h to lay eggs, after which these females were also discarded. These vials were kept in temperature-controlled rooms for 12 more days to allow any fertilized eggs to develop into adults. The insemination capacity of each focal male was measured as the number of tester females (a maximum of 10 per male) producing pupae.

We also analyzed published data on competitive male fertility within the LH_M population; an outbred, laboratory-adapted population (Innocenti and Morrow 2010). Innocenti and Morrow (2010) generated hemiclones (genetically identical for half of the diploid genome, Abbott and Morrow 2011) and screened them for total adult lifetime fitness. This was done in a competitive assay environment, where five males per hemiclone genotype were tested with 10 competitor males (with bw^- brown eye color markers) and 15 virgin bw^- females for two days. Females were then separated from males and allowed to lay eggs for 18 hours. The progeny were scored for eye colour to assign paternity to the hemiclone (bw^+/bw^- offspring) or competitor (bw^-/bw^- offspring) males to obtain a measure of relative adult male fitness. This assay was replicated six times per hemiclone genotype. Phenotype data are available from (www.sussex.ac.uk/lifesci/morrowlab/data).

GENE EXPRESSION

Gene expression data were downloaded from Huang et al. (2015), comprising two replicates per sex for each DGRP line (n_{genes} = 18,140). These data were the summarised gene expression data (http://dgrp2.gnets.ncsu.edu/data.html) preprocessed from Illumina TruSeq mRNA-seq. Briefly, data consisted of 25 pooled female flies or 40 pooled male flies per replicate per DGRP line. Therefore, for each DGRP line we had the estimated average level of expression for males and females. Sex-bias for each gene was calculated as the $\log_2$ fold change between the average expression across all males divided by the average expression across all females ($\log_2$ fold change male:female). Full methods can be found in Huang et al. (2015).

For the LH_M dataset (Innocenti and Morrow 2010), gene expression data were measured using microarrays, from four replicates per hemiclone per sex. Sex-biased gene expression was analyzed in the same way to the analyses described above for the DGRP lines.

STATISTICAL ANALYSES

Genetic variation for male quality

We first determined whether we could detect genetic variation for our different measures of male reproductive quality across the DGRP lines. Latency to copulate was log transformed to approximate a normal distribution. Log-transformed latency to copulate was fitted with a linear-mixed model and REML algorithm using the lme4 package (Bates et al. 2012) in R v. 3.3.1. An intercept of 1 was specified, and block and DGRP line were specified as random factors (model: male quality measure = 1 + $\text{block}_{(\text{random})}$ + DGRP $\text{line}_{(\text{random})}$). Log-likelihood ratios tests were used to assess statistical significance (at $P < 0.05$) for the random factors by quantifying change in deviance when removing each random effect from the model.

Male insemination capacity was fitted with a generalized linear model, an intercept of 1 and Poisson error distribution (model: male insemination capacity ~ 1). The model was then tested for underdispersion using the AER package (Kleiber and Zeileis 2008) in R. Since the data were underdispersed (dispersion estimate = 0.63, $z = -4.7$, $P < 0.0001$), a GLMM model using Penalized Quasi-Likelihood (PQL) and quasipoisson error distribution was fitted using the MASS package (Venables and Ripley 2002) in R (model: male quality measure = 1 + $\text{block}_{(\text{random})}$ + DGRP $\text{line}_{(\text{random})}$). Log-likelihood ratio tests are not supported for PQL fits since they require an optimisation criterion (Venables and Ripley 2002), and as such we do not provide *P*-values for the random effects for male insemination capacity. To test whether our two measures of male quality were correlated, we ran a linear regression between male insemination capacity and latency to copulate.

Calculation of genetic covariance and heritability

We used a mixed-effect model to estimate the heritability of male reproductive phenotypes using MCMCglmm v2.24 (Hadfield 2010). Log transformed latency to copulate was modelled with a Gaussion error distribution with Block as a fixed effect and DGRP line as a random effect. We specified the prior for the residual and random effects variances as 0.002, which is weakly informative for small sample sizes with larger variances. We specified the default priors for the fixed effects. Two independent MCMC chains (Griffith et al. 2016) were run for 250,000 iterations with a burn-in of 75,000. Convergence was visually checked using trace plots and autocorrelation scores. The distribution of heritability values was taken as the ratio of the posterior distributions of the additive (VA) and phenotypic (VP) variances with the mean giving our heritability estimate for each phenotype.

For male insemination capacity an ordinal error distribution was specified with Block as a fixed effect and DGRP line as a random effect. Residual and random variances were fixed at 1. Iterations were increased to 25,000,000 with a burn-in of 5,000,000 however models failed to converge.

Associations between male quality and gene expression

Since long latencies to copulate denote lower quality males, we transformed this measure (Inverse latency to copulate = 1/latency to copulate). This means that high values equate to males that were quick to copulate and low values equate to males that were slow to copulate, facilitating clearer comparison between the two male quality measures. We next scaled each male quality measure to have mean of zero and standard deviation of one to facilitate comparison between the measures.

For each gene, Spearman's rho correlation coefficient (ρ) was calculated between the average phenotypic measure of male quality (for each of the two traits) per genotype and the estimated average level of gene expression for males in each DGRP line ($n_{genotypes}$ = 33, model: Average male quality ~ Average expression of gene_1). For each gene, we therefore had a value of ρ that measures the rank order correlation between phenotype and gene expression.

Next, we tested how sex-biased gene expression affected the direction and strength of the relationship between male quality phenotype and gene expression. We analyzed the relationship between ρ and sex-bias in gene expression as a continuous variable (i.e., $\log_2$ fold change in expression for males:females). For illustration purposes, we plotted mean ρ for 0.1 increments of sex-bias (Mank et al. 2008; Dean and Mank 2016), weighting the size of each data point by the number of genes in each increment. We analyzed this relationship in two ways. First, we analyzed the rank order monotonic relationship between ρ and sex-bias across all genes using Spearman's rank correlation. Second, because

male-biased and female-biased genes may show different relationships with male phenotype we split genes into male-biased (i.e., those with more than twice the expression in males compared to females, i.e., $\log_2$ male:female >1) and female-biased (i.e., those with more than twice the expression in females compared to males, that is $\log_2$ male:female <-1). We then tested for linear and quadratic relationships between ρ and sex-bias for male- and female-biased genes and plotted the model-fitted line. If the quadratic relationship was nonsignificant, correcting for multiple testing (adjusted $\alpha = 0.00125$, (at $P = 0.01$, with eight different tests)), we present the linear model.

We also ran linear-mixed effects models to test whether the expression of male-biased genes was more positively correlated with male phenotype than female-biased genes. Using lmer in R (Bates et al. 2015), we specified the model: phenotype ~ gene expression × sex-bias + (1|geneID), where each data point is a DGRP line (such that there are $n_{lines} \times n_{genes}$ (i.e., 33 × 18,140) data points, and n_{genes} repeated measures of each line). The number of iterations was increased to 50,000. The fitted model lines for male-biased, female-biased, and unbiased genes were plotted using the Effects package (Fox 2003) in R. We also ran separate models for male-biased, female-biased, and unbiased genes using the model: phenotype ~ gene expression + (1|geneID).

Sex chromosomes and associations between gene expression and male quality

Because the sex chromosomes contain an excess or deficit of sex-biased genes (Parisi et al. 2003; Ranz et al. 2003), we next identified genes on the X and Y chromosomes. Analyses for the association between ρ and sex-bias were repeated, splitting genes up based on their chromosomal location on the X chromosome or the autosomes. We also looked at the relationship between gene expression and male phenotype for individual genes on the Y chromosome.

GWA to identify SNPs that associate with male quality

A GWA using the DGRP resource (Mackay et al. 2012; Huang et al. 2014) was run to identify SNPs that associate with male quality. Since many SNPs are in high linkage with each other, we implemented a SNP clumping approach using bigsnpr package in R (Privé et al. 2018). After filtering out SNPs based upon missing genotypes and low minor allele frequency (<0.05), SNPs were clumped together if they had $r^2 > 0.05$ (estimated from Fig. 1C Mackay et al. 2012). Out of the 1.2 million SNPs that were tested for our 33 DGRP lines, this left 715 SNPs that were not in linkage (see supplementary methods for more information on quality control thresholds). Next, the *P*-values from the GWAS regression were adjusted for multiple testing using the FDR method and SNPs that significantly associate with the male phenotypes at the level of $P_{adj} < 0.05$ are reported in the supplementary information. We conducted a power analysis using the pwr package in R (Champely 2017) to test the power to detect an association between a single SNP and the male phenotypes (i.e., not simultaneously testing all 1.2 millions SNPs) specifying a sample size of 33, an effect size of 0.1, and significant level of 0.05.

Associations between male quality and gene expression for LH_M population

We also analyzed the association between male gene expression and male quality, as measured by competitive male fertility, in the LH_M dataset (Innocenti and Morrow 2010). This dataset consists of 15 hemiclones, specifically chosen out of a population of 100 hemiclones, for their sexually antagonistic fitness. These 15 hemiclones consist of five lines with high male fitness and low female fitness, five lines with low male fitness and high female fitness, and five lines with intermediate fitness in both males and females, where fitness was defined using the competitive male fertility assay described previously. As before, Spearman's rho rank order correlation (ρ) was calculated, per gene, between male gene expression and male phenotype ($n_{hemiclones} = 15$). The association between ρ (i.e., the correlation between gene expression and phenotype), and sex-biased gene expression was analyzed in the same way as for the DGRP dataset.

All analyses were performed in R (v.3.3.1) (R-Core-Team 2016).

Results

PHENOTYPIC ASSOCIATIONS WITH MALE QUALITY

Across the 33 inbred lines, we detected significant genetic variation for latency to copulate (Table 1, Fig. 1). For male insemination capacity, we are unable to use the log-likelihood ratio test on PQL fits; however, standard deviations for DGRP line and block are presented in Table 1. For latency to copulate, VA = 0.054 (SD = 0.017) and VP = 0.176 (SD = 0.019). Heritability for latency to copulate was 0.30 (SD = 0.066, 95% CI 0.19-0.44). There was no association between male insemination capacity and copulation latency (Fig. 2).

ASSOCIATIONS BETWEEN MALE QUALITY AND MALE GENE EXPRESSION

No individual gene showed a significant Spearman's ρ correlation between male expression level and male quality phenotype after FDR correction for multiple testing, for either of the male quality phenotypes measured.

Latency to copulate

There was a significant monotonic relationship between ρ (i.e., the rank order correlation between gene expression and latency to copulate) and sex-biased gene expression (Fig. 3A, Spearman's

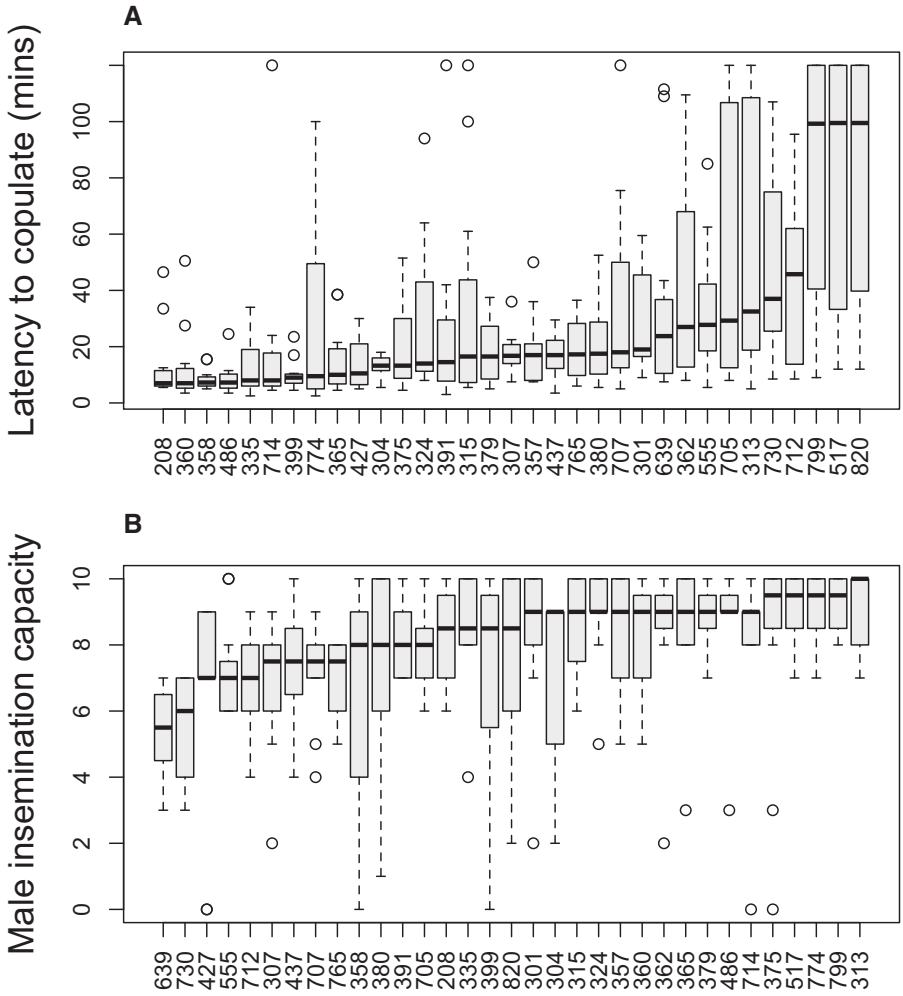

Figure 1. Variation in male quality measures across the DGRP lines (A) latency to copulate and (B) number of females inseminated. Boxes represent medians and first and third quartiles.

Table 1. Contribution of genotype (DGRP line) and block effect to variance in male quality measures.

Measure	Factor	S.D	LRT	df	*P*
Copulation latency[a]	DGRP line	0.23	76.1	1	**<0.0001**
	Block	0.05	2.28	1	0.131
	Residual	0.35			
Insemination capacity[b]	DGRP line	0.10	–	–	–
	Block	0.08	–	–	–
	Residual	0.74			

[a]Log_{10} transformed copulation latency (linear-mixed model).

[b]Quasi-Poisson distribution (generalized linear-mixed model with Penalized Quasi-Likelihood).

Models are fitted with DGRP line and Block as random factors. Log-likelihood ratios tests (LRT) generated *P*-values for the random factors (linear-mixed model only).

rho = 0.576, $P < 0.0001$), such that the rank order of ρ increases with increasing sex-bias.

More specifically, dividing genes into male-biased and female-biased revealed a significant quadratic relationship for male-biased genes (Fig. 3A, Estimate = –0.01, $F_{2,71} = 266$, $P <$ 0.0001), such that as genes become more male-biased ρ increases and then declines. These results suggest that higher expression of male-biased genes confers a higher quality male phenotype (i.e., shorter latency to copulate) up until extreme male-biased expression of around $\log_2$ fold change male:female >5.

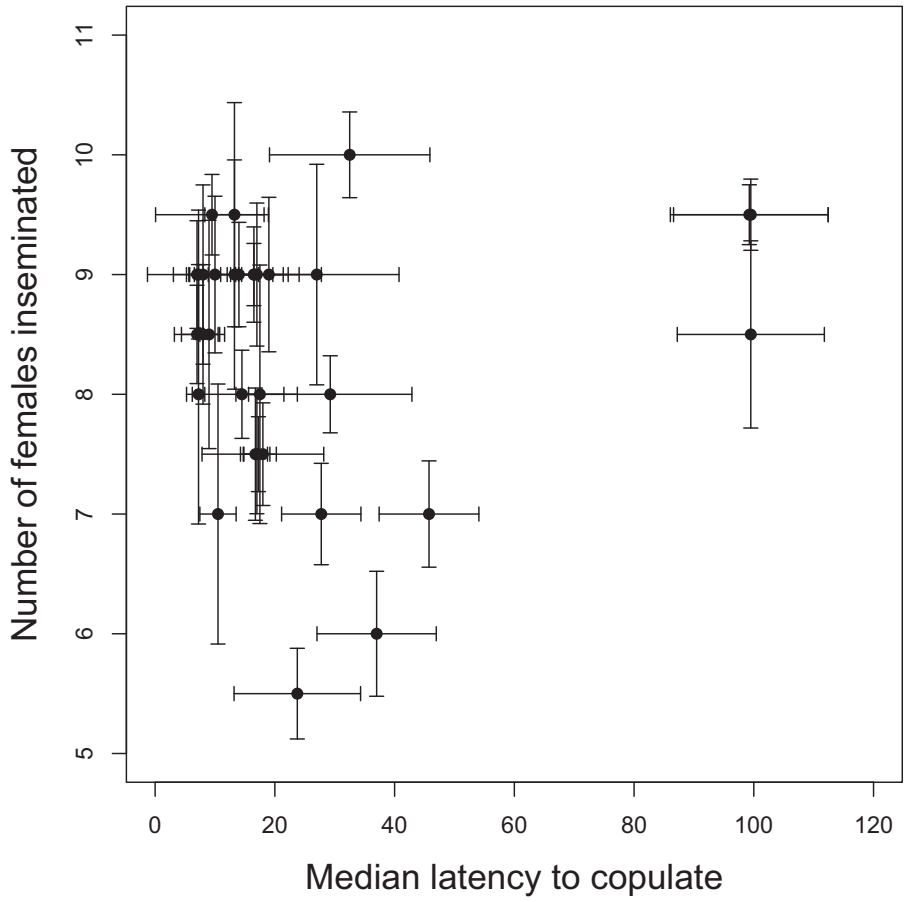

Figure 2. Relationship between the different measures of male quality. Error bars denote median ± standard error. Linear model between latency to copulate and number of females inseminated, $F_{1,31} = 0.26$, $P = 0.61$.

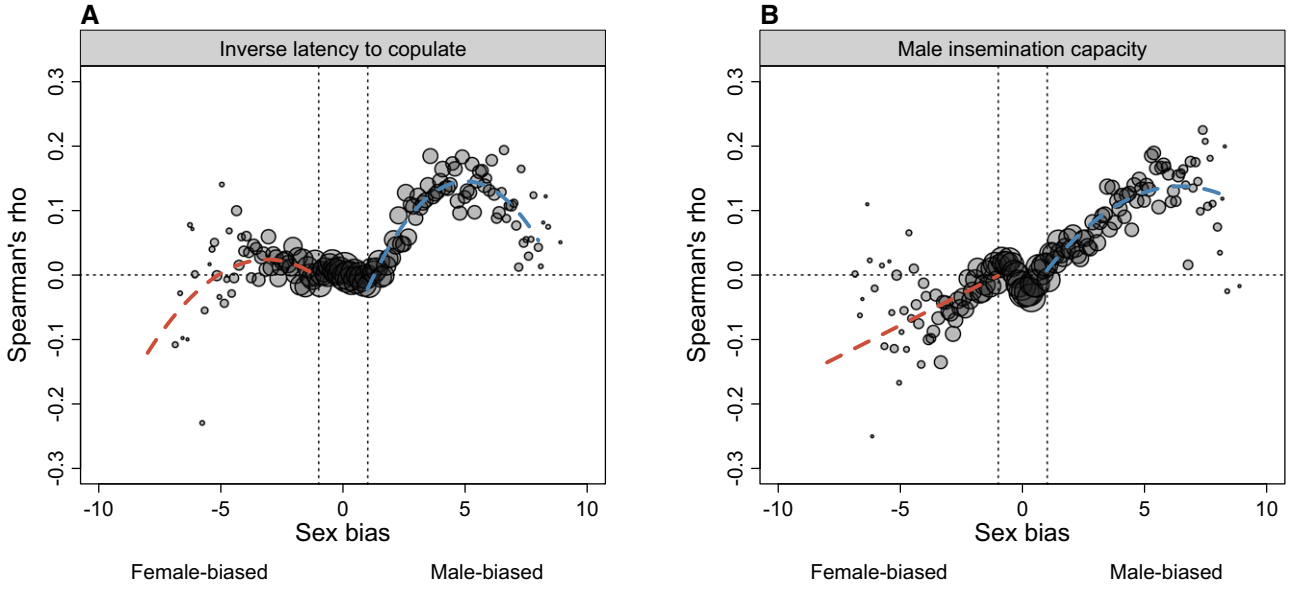

Figure 3. Sex-biased gene expression (log_2 male:female) and Spearman's rho correlation coefficient between phenotype and gene expression per gene for (A) inverse latency to copulate and (B) male insemination capacity. Size of data point scales with number of genes in each bin. Dashed lines represent significant model predictions, with male-biased genes in blue and female-biased genes in red.

There was also a significant quadratic relationship for female-biased genes (Fig. 3A, Estimate = –0.006, $F_{2,60} = 8.0$, $P = 0.0002$). As genes get more female-biased, ρ increases moderately and then declines for genes with more extreme degrees of female-bias. In other words, high expression of weakly female-biased genes confers male reproductive advantage, but high expression of extremely female-biased genes confers a lower quality male phenotype (i.e., long latency to copulate).

Male insemination capacity

There was a significant monotonic relationship between ρ (i.e., the relationship between gene expression and male insemination capacity) and sex-biased gene expression (Fig. 3B, Spearman's rho = 0.714, $P < 0.0001$) such that the rank order of ρ increases with increasing sex-bias.

Dividing genes into those with male-biased and female-biased expression revealed a linear relationship for female-biased genes (Fig. 3B, Estimate = 0.02, $F_{1,61} = 37.0$, $P < 0.0001$) and a curvilinear relationship for male-biased genes (Fig. 3B, Estimate = –0.004, $F_{2,77} = 114$, $P < 0.0001$). In other words, for male-biased genes, higher expression in males equates to a high insemination capacity. This relationship levels out for extremely male-biased genes. For female-biased genes, high expression in males equates to low male insemination capacity.

Linear-mixed model approach

We also ran linear-mixed effects models to test whether the expression of male-biased genes was more positively correlated with male phenotype than female-biased genes. We found significant interactions between sex-bias and male gene expression for latency to copulate and male insemination capacity (Fig. 4A, B). For male-biased genes, gene expression was positively associated with inverse latency to copulate (Fig. 4A, Estimate ± Standard error = 0.0061 ± 0.0017, d.f = 1, F-ratio = 13.45, $P = 0.0002$) and positively associated with male insemination capacity (Fig. 4B, Estimate ± Standard error = 0.0080 ± 0.0017, d.f = 1, F-ratio = 23.11, $P < 0.0001$).

For female-biased genes, gene expression was not associated with inverse latency to copulate (Fig. 4A, Estimate ± Standard error = 0.0005 ± 0.0021, d.f = 1, F-ratio = 0.045, $P = 0.829$) and was negatively associated with male insemination capacity (Fig. 4B, Estimate ± Standard error = –0.0050 ± 0.0021, d.f = 1, F-ratio = 5.47, $P = 0.0193$).

For unbiased genes, gene expression was not associated with inverse latency to copulate (Fig. 4A, Estimate ± Standard error = –0.0004 ± 0.0007, d.f = 1, F-ratio = 0.265, $P = 0.607$) and was negatively associated with male insemination capacity (Fig. 4B, Estimate ± Standard error = –0.0017 ± 0.0007, d.f = 1, F-ratio = 5.99, $P = 0.0144$). Our results reveal qualitatively similar patterns using the two different analytical approaches.

SEX CHROMOSOMES AND ASSOCIATIONS BETWEEN MALE GENE EXPRESSION AND MALE QUALITY

We next tested for associations between male gene expression on the sex chromosomes and male quality. For inverse latency to copulate, genes on both the autosomes (Fig. 5A, rho = 0.548, $P < 0.0001$) and the X chromosome (Fig. 5A, rho = 0.307, $P = 0.0004$) showed significant monotonic relationships.

This relationship was driven by male-biased genes on both the autosomes (Estimate = –0.011, $F_{2,77} = 232$, $P < 0.0001$) and X chromosome (Estimate = –0.012, $F_{2,54} = 11.2$, $P = 0.0007$). There was no significant relationship between ρ and sex bias for female-biased genes on the autosomes or X chromosome.

Similarly, for male insemination capacity, genes on both the autosomes (Fig. 5B, rho = 0.685, $P < 0.0001$) and the X chromosome (Fig. 5B, rho = 0.642, $P < 0.0001$) showed significant monotonic relationships. This relationship was driven by genes on the autosomes for both male-biased (autosomes: Estimate = –0.004, $F_{2,77} = 96.8$, $P < 0.0001$) and female-biased genes (autosomes: Estimate = 0.02, $F_{1,56} = 36.3$, $P < 0.0001$). There was no significant relationship for sex-biased genes on the X chromosome.

No genes on the Y chromosome had a significant association between male gene expression and male phenotype, for either of the male quality phenotypes measured.

ASSOCIATIONS BETWEEN MALE QUALITY AND GENE EXPRESSION FOR A DIFFERENT *DROSOPHILA* POPULATION

Finally, we repeated the analysis for a different population of *D. melanogaster* (LH_M), using the dataset of Innocenti and Morrow (2010). In this dataset, male quality across a set of hemiclonal lines was measured as male reproductive success when five focal hemiclonal males competed against ten competitor males over 15 females (Innocenti and Morrow 2010). There was a significant monotonic relationship between ρ and sex-bias (Fig. 6, rho = 0.586, $P < 0.0001$). Male-biased genes showed a significant quadratic increase in ρ as sex-bias increases (Estimate = 0.005, $F_{2,87} = 40.3$, $P < 0.0001$). Female-biased genes showed no quadratic or linear relationship between ρ and sex-bias. In other words, there was no relationship between male phenotype and expression of female-biased genes, but higher expression of male-biased genes confers higher male reproductive success and lower expression of male-biased genes confers a lower male reproductive success.

GENOME-WIDE ASSOCIATION ON MALE QUALITY MEASURES

We conducted a GWA to detect SNPs that associate with our male phenotypes. After correcting for linkage disequilibrium using SNP clumping (Privé et al. 2018), the GWA found one SNP

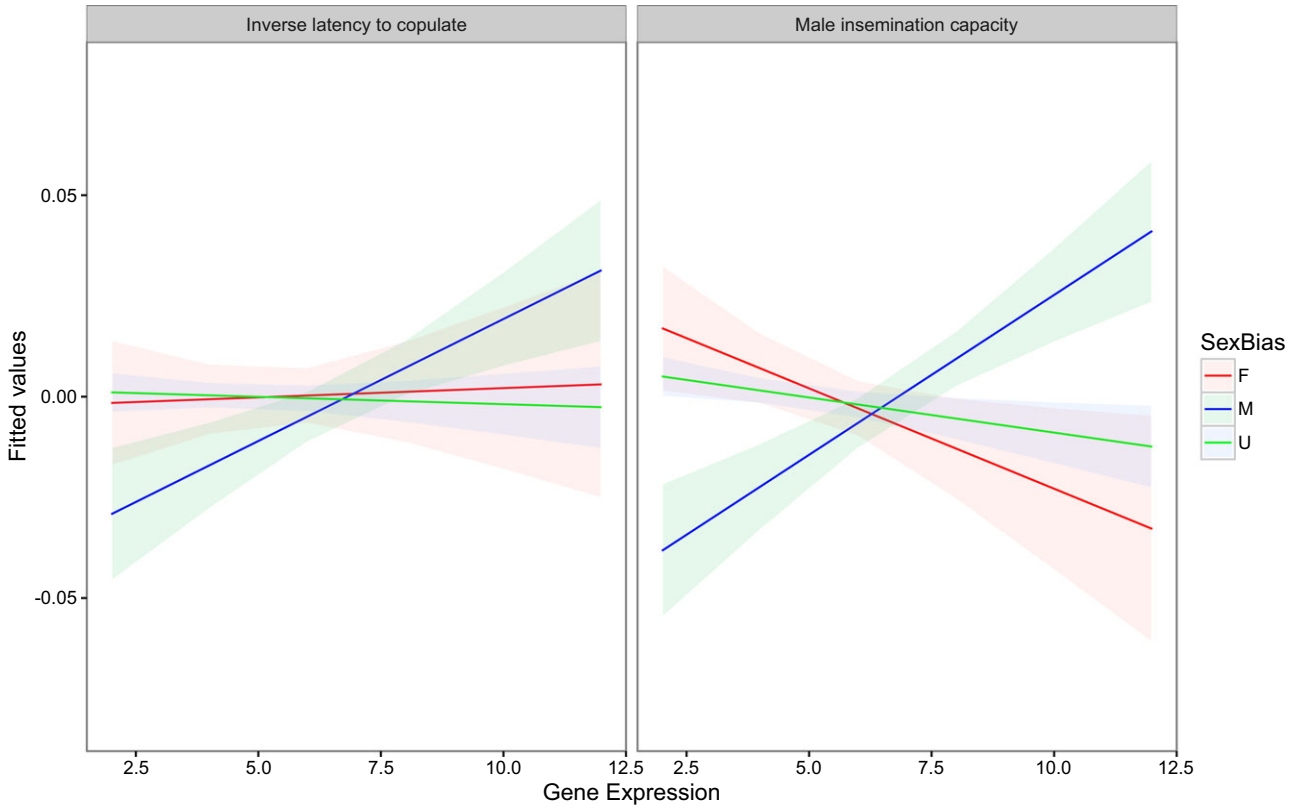

Figure 4. Model predictions for the relationship between phenotype and gene expression for (A) inverse latency to copulate and (B) male insemination capacity. Female-biased genes (F) in red, male-biased genes (M) in blue and unbiased genes (U) in green. For both phenotypes there were significant interactions between sex-bias and gene expression. Inverse latency to copulate: sex-bias × gene expression, d.f = 2, F-ratio = 6.38, $P = 0.0017$. Male insemination capacity: sex-bias × gene expression, d.f. = 2, F-ratio = 16.77, $P < 0.0001$.

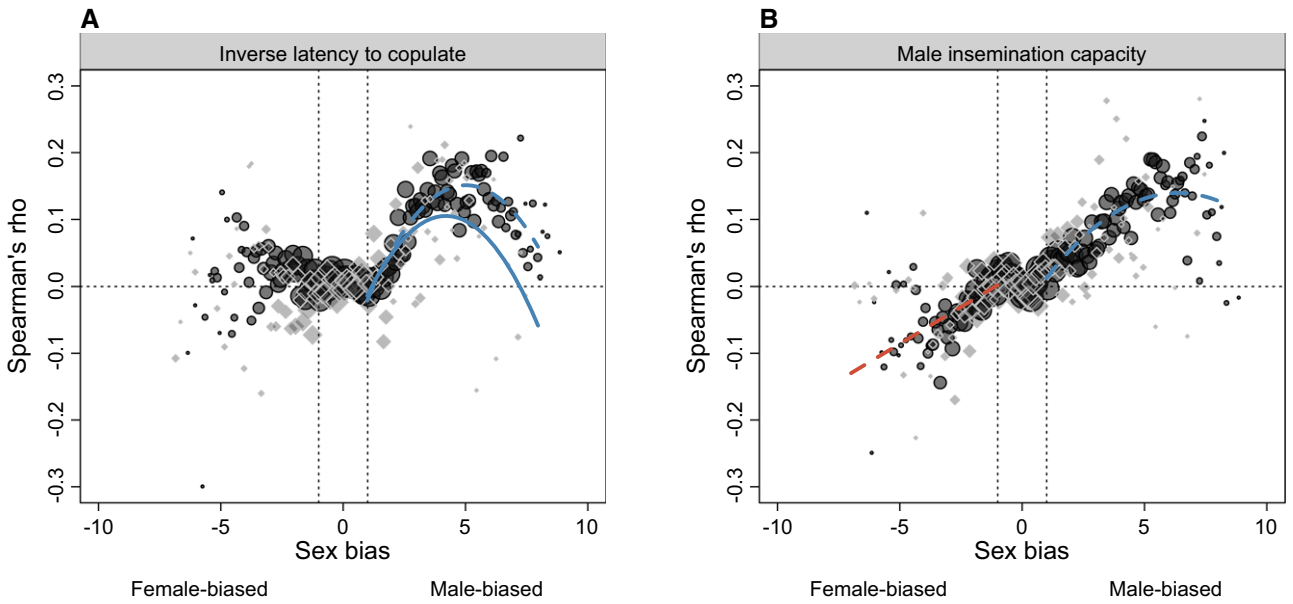

Figure 5. Sex-biased gene expression (log_2 male:female) and Spearman's rho correlation coefficient between phenotype and gene expression per gene for (A) inverse latency to copulate and (B) male insemination capacity. Size of data point scales with number of genes in each bin. Black circles and dashed lines are for autosomal genes and gray diamonds and solid lines are for genes on the X chromosome. Lines represent predicted models, with male-biased genes in blue, and female-biased genes in red.

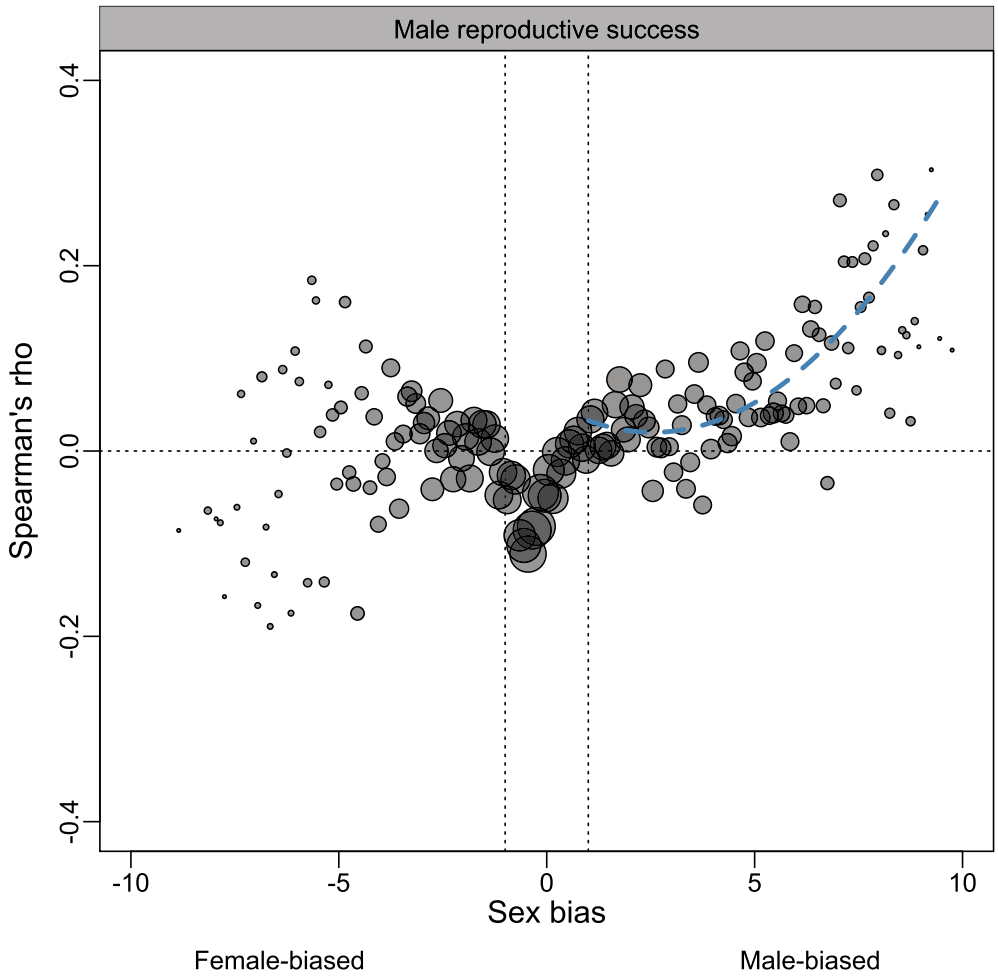

Figure 6. Sex-biased (log_2 male:female) gene expression and Spearman's rho correlation coefficient between male reproductive success and male gene expression per gene in Drosophila from the LH_M population and using data from Innocenti and Morrow (2011). Size of data point scales with number of genes in each bin. Dashed line represents models predictions for male-biased genes.

on the X chromosome that associated with copulation latency (Table S1 in supplementary material) and no SNPs that associated with male insemination capacity, following FDR correction for multiple testing at the threshold of 0.05. However, a power analysis showed that our GWA had only a small (13%) chance of detecting SNPs with a 10% effect on the fitness phenotype, suggesting that our study lacks power and is likely to have missed many SNPs with a small effect on male phenotype.

Discussion

Gene expression studies of alternative male mating tactics have been used to study how sexual dimorphism in phenotypes scales with sexual dimorphism in gene expression, and to investigate how sperm competition intensity shapes patterns of male-biased gene expression within the gonads (Pointer et al. 2013; Dean et al. 2017). However, whether these patterns of masculinisation of gene expression extend to species without distinct male morphs has remained untested. Here, we explored whether variation in expression levels of male-biased genes associates with variation in components of male reproductive quality, in *D. melanogaster*, a species lacking clear alternative male mating tactics. We used two different populations of flies, each of which captures genetic variation within the population through the use of inbred lines (fully isogenic diploid genomes) or hemiclonal lines (isogenized haploid genomes placed alongside a randomized haploid genome).

In the DGRP population, we found that as sex-biased gene expression becomes more male-biased, genes showed stronger associations (more positive Spearman's ρ) between gene expression and phenotype. This was the case for both components of male quality; male latency to copulation, and male insemination capacity. We also found positive slopes between gene expression and male phenotype for male-biased genes for both phenotypes. Thus, for male-biased genes, higher

expression in males is associated with a higher quality male phenotype. For female-biased genes, lower expression in males is associated with a high quality male phenotype; however, the shape of these relationships was different across the two phenotypes measured.

Surprisingly, we found associations between male phenotype and gene expression peaked at intermediate levels of male-bias (i.e., $\log_2$ fold change male:female $\sim$ 5), rather than following a linear relationship. Although we are unable to ascertain why strongly sex-biased genes do not show strong associations with male phenotype, we can speculate about the causes. One reason may be that sexual conflict may have been resolved through past sexually antagonistic selection for strongly sex-biased genes, meaning that current expression variation is no longer antagonistic (Rowe et al. 2018). In line with this, a similar pattern has been shown in human and fly populations that looked at sex-biased gene expression and $F_{ST,}$ a measure of genetic divergence between males and females due to differences in viability selection (Cheng and Kirkpatrick 2016). This study showed that genes with intermediate sex-bias are targets of strongest sex-specific selection and that genes with either strong or weak sex-bias are under weaker sex-specific selection (Cheng and Kirkpatrick 2016). This may explain why, in our study, genes with intermediate sex-bias associate most strongly with male quality phenotype. It is important to note, however, that sex-specific selection resulting from differences in reproductive fitness may reveal different patterns (Wright et al. 2018).

For male reproductive success in the LH_M population, the pattern for male-biased genes was similar to the DGRP population, with higher expression of male-biased genes linked to higher male reproductive success. However, in this population no relationship was found for female-biased genes. This convergence on similar patterns between the two populations (DGRP and LH_M) for male-biased genes is striking, given that the male quality traits measured in each of the populations differed in an important way. In our assays of the DGRP dataset, we measured traits that reflected precopulatory components of reproductive success (latency to copulation and capacity to secure matings with multiple females). However, the male quality measure in the LH_M population (paternity success when competing against rival males for fertilizations of multiple females) will have been shaped by both pre- and postcopulatory components, as well as by stochastic environmental factors associated with the experimental design, such as the timing of which males procured the final mating with a given female prior to egg laying (given the strong second male sperm precedence in *D. melanogaster*). This suggests that the patterns we have uncovered between levels of male-bias in gene expression and male reproductive quality are general across the gamut of male reproductive traits, including those under precopulatory and postcopulatory selection.

MAINTENANCE OF GENETIC VARIATION

It is thought that genetic variation for male sexually selected traits is maintained within populations, despite strong directional selection (the lek paradox, Taylor and Williams 1982; Kirkpatrick and Ryan 1991), due to condition-dependence (Rowe and Houle 1996; Tomkins et al. 2004), and/or a large number of loci contributing to condition (Rowe and Houle 1996). The male quality traits we measured in our study are likely to be highly condition-dependent given they reflect male sexual behaviors, which may result in a large environmental influence on our trait measures, thus reducing the capacity to have detected genotypic associations between the traits and patterns of gene expression at the sample sizes used. Although some of the DGRP lines showed substantial variation in male quality measures across the different replicates and blocks (Fig. 1), suggesting that environmental variation plays a role in determining trait values, our analyses show that genotype contributed to variation in male quality measures to a greater extent than variation across blocks (Table 1). Furthermore, we note that we attempted to carefully control for potential environmental sources of variation from affecting our results, through regulating egg densities of clutches that produced the focal males and tester females, standardizing the ages of the parental flies that produced the focal and tester flies, and standardizing both the dietary and thermal conditions.

The second component explaining maintenance of genetic variation in male sexually selected traits relates to many loci contributing to trait expression (Rowe and Houle 1996). In line with this, our results are consistent with the expression of many male-biased genes, each with small effect, contributing to male quality, rather than a few candidate genes whose expression strongly correlated with male quality. While relationships between sex-biased gene expression and individual quality have previously been reported in species with alternative male morphs (Pointer et al. 2013; Dean et al. 2017), our study reveals that similar transcriptomic patterns determine variation in male quality within a population, in a species that does not exhibit clearly divergent male morphs.

SEX CHROMOSOMES

Due to their asymmetric patterns of inheritance and difference in copy number between the sexes, the sex chromosomes are expected to play a role in encoding sex differences (Mank 2009). It is well established that the sex chromosomes harbor a nonrandom distribution of sex-biased genes, but whether these sex-biased genes play a key role in contributing to sex differences in phenotypic expression is less well understood (Beukeboom and Perrin 2014; Dean and Mank 2014). The X chromosome in *Drosophila* contains an excess of female-biased genes (Ranz et al. 2003) and few strongly male-biased genes (Parisi et al. 2003), and thus we may not expect X-linked male-biased genes to reflect variation in male quality to the same extent as male-biased genes across

the whole of the genome. Accordingly, the relationship between expression of male-biased genes and male quality was less pronounced for X-linked genes. On the other hand, the Y chromosome experiences strictly paternal transmission, and should be a prime location for genes that affect male reproduction (Lahn and Page 1997). However, we did not find any association between variation in expression of Y-linked genes and male reproductive quality. Notwithstanding, the Y chromosome also exerts a large regulatory role on the rest of the genome, affecting the expression of hundreds to thousands of autosomal genes (Lemos et al. 2008), and thus the true influence of the Y chromosomes to encoding the male reproductive phenotypes is likely to extend well beyond the contribution of the few protein-coding genes located on it.

To conclude, we found that higher expression of male-biased genes is associated with variation in male phenotypes associated with the outcomes of reproduction. Since we did not identify specific genes whose expression correlated with these reproductive phenotypes, it is likely that male reproductive quality is underpinned by the concerted action of many genes of small effect. Notably, the patterns we revealed were consistent across two different *D. melanogaster* populations, and across a diverse set of reproductive traits; those shaped primarily by precopulatory sexual selection, and those shaped by pre- and postcopulatory selection. This indicates that the transcriptomic patterns that we have uncovered are likely to reflect pervasive responses to selection on males, at least among *Drosophila*.

AUTHOR CONTRIBUTIONS

R.D., D.K.D. designed the research, R.D., C.H., V.H. collected data, R.D. analyzed data, R.D., D.K.D. wrote article.

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DATA ARCHIVING

Phenotypic data for the DGRP dataset archived at Dryad at https://doi.org/10.5061/dryad.s3mr73b. DGRP expression data are available from http://dgrp2.gnets.ncsu.edu/. Phenotypic data from the LH_M population is available at www.sussex.ac.uk/lifesci/morrowlab/data and expression data are on the GEO database, accession number GSE17013.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. SNPs that associate with the male quality phenotypes.