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# Genetic constraints on microevolutionary divergence of sex-biased gene expression

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The evolution of sex-specific phenotypes is an important dimension of diversification and local adaptation. The sex-dependent regulation of gene expression is considered a key genomic mechanism facilitating sex-dependent adaptation. In many species, genes with male-biased expression evolve faster in DNA sequence and expression level than genes with female-biased or sexually monomorphic expression. While positive selection may be responsible for rapid DNA sequence evolution, why expression of male-biased genes also evolves rapidly remains unclear. Beyond sex differences in selection, some aspects of the genetic architecture of gene expression could contribute to the rapid evolution of male-biased gene expression. First, male-biased genes might simply have greater standing genetic variance than female-biased genes. Second, male-biased genes could be less constrained by pleiotropy, either within or between sexes. Here, we evaluate these alternative explanations on an intraspecific scale using a series of quantitative genetic experiments conducted on natural variation in male and female gene expression in the fly *Drosophila serrata*. Male-biased genes had significantly higher genetic variance than female-biased genes and were generally more narrowly expressed across tissues, suggesting lower within-individual pleiotropy. However, consistent with stronger constraints due to between-sex pleiotropy, their between-sex genetic correlations, $r_{MF}$, were higher than for female-biased genes and more strongly negatively associated with sex bias. Using an extensive clinal dataset, we tested whether sex differences in gene expression divergence among populations have been shaped by pleiotropy. Here too, male-biased gene divergence was more strongly associated with between-sex pleiotropy than was female-biased gene divergence. Systematic differences in genetic variance and pleiotropy may be important factors influencing sex-specific adaptation arising through changes in gene expression.

This article is part of the theme issue 'Linking local adaptation with the evolution of sex differences'.

## 1. Introduction

Sexually dimorphic gene expression is common in species with two sexes [1–3] and is considered a key mechanism by which sexually dimorphic traits can be produced from a common genome [1,4]. Although multiple mechanisms can cause sex-biased expression, it is thought that many sex-biased genes have become so due to historical selection favouring different trait optima in males and females [5]. Selection for sexually dimorphic traits can result from resource competition favouring ecological niche partitioning between the sexes [6–8], or from sex-specific reproductive strategies that favour distinct behaviour, morphology or physiology in females and males [9,10]. In both cases, sex-specific trait diversification is likely to be closely linked to ecological parameters that shape niches and reproductive strategies. The evolution of sex-biased gene expression could therefore play an important role in the adaptation and diversification of sexual lineages.

While sexual dimorphism is widespread, many sex-biased genes seem to experience ongoing selection for divergence between the sexes [11,12]. For example, by eliminating major components of selection on male *Drosophila melanogaster* and allowing selection to occur on females over multiple generations, female-biased genes became more female-biased, while male-biased genes became less male-biased [13]. Essentially, female-limited selection 'feminized' the transcriptome, indicating the presence of current sexually divergent selection. The detection of ongoing sexually divergent selection, even though some degree of sex bias has evolved, is a strong indication that the evolution of sex bias is constrained such that neither sex is expressing genes at its optimal level [14,15]. Yet, despite these apparent constraints, male-biased genes are some of the fastest evolving genes across a diverse range of species [1,2,16], in terms of protein coding sequence [17,18] and gene expression level [1]. Such evidence suggests that male traits might be more evolvable than female traits, perhaps giving males an advantage in sexually antagonistic evolution or enabling males to escape resource competition with females by evolving to exploit a different ecological niche. Understanding how sexual lineages evolve and diversify therefore requires understanding the factors that influence evolution of male-biased and female-biased genes.

Here, we evaluate several possible explanations for the accelerated evolution of male-biased gene expression that are yet to be explored. The first is a simple quantitative genetic explanation. As the response to selection in any trait is proportional to its genetic variance [19,20], it is possible that male-biased gene expression evolves faster because male-biased genes harbour more genetic variance. Under such a scenario, faster divergence of male-biased gene expression is expected even if selection strength does not differ across sex bias categories. Indeed, across 122 studies spanning a broad range of species and phenotypes, there was a skew towards males having more genetic variance than females and the degree of sexual dimorphism in genetic variance was positively correlated with the degree of phenotypic sexual dimorphism [21]. Perhaps this extends to genetic variance in expression of male-biased genes relative to female-biased genes.

Second, male-biased genes may evolve faster than other classes of gene because they are less constrained by pleiotropy [14,18,22–25]. Pleiotropy could constrain evolution because of widespread multivariate stabilizing selection, where, for example, an increase in expression of a gene could shift some traits closer to their optima while simultaneously pulling other traits away [26–29]. The extent to which a gene is pleiotropic has previously been inferred from a quantitative measure of tissue specificity ($\tau$) [14,18,22,30]. Genes expressed widely across multiple organs and tissue types (low $\tau$) more likely affect many different phenotypic traits, compared with those expressed in a limited number of tissues (high $\tau$). If pleiotropy constrains the evolution of sex-biased expression, a positive correlation between $\tau$ and the degree of sex bias is expected and would indicate that the most sex-biased genes tend to be the least broadly expressed and therefore least pleiotropic. This has been observed in a broad range of species, including mice (*Mus musculus*), chickens (*Gallus gallus*) [14] and flies (*D. melanogaster* and *Drosophila pseudoobscura*) [18]. While these patterns are a clear sign that pleiotropy can influence the evolution of sex-biased gene expression, it remains untested whether the relationship is weaker for male-biased genes than for female-biased genes. Such a result could help explain why male-biased genes often evolve faster than female-biased genes.

Because males and females share a genome, pleiotropy between sexes can also slow sex-specific evolutionary responses [27,31]. The strength of between-sex pleiotropy can be estimated via the between-sex genetic correlation, $r_{MF}$. Large positive values of $r_{MF}$ indicate a strong contribution to genetic (co)variance between sexes due to pleiotropic alleles with similar effects on males and females. In such cases, the evolution of sexual dimorphism will be far slower than when $r_{MF}$ is weak. A negative correlation between $r_{MF}$ values and the degree of sexual dimorphism across traits has been used to infer between-sex pleiotropic constraints on the evolution of sexual dimorphism [32,33]. Application of this method to gene expression data has revealed mixed results, with genetic constraints detected in *D. melanogaster* [15] and birds [34], but not in human blood, for which expression dimorphism is generally quite low [35]. While these studies suggest that a shared genome could constrain the evolution of sex-biased expression, it again remains unclear whether the strength of between-sex constraints differs between male- and female-biased genes.

While most evidence for differences in evolutionary rates of male- and female-biased gene expression levels comes from interspecific studies, some microevolutionary studies in *Drosophila* have found similar patterns [36–38]. We recently showed that many more male- than female-biased genes have diverged in expression level among eight natural populations of *Drosophila serrata* that span a latitudinal gradient [39]. Notably, the magnitude of divergence in these male-biased genes was greater when measured in males than when measured in females. Furthermore, despite apparent sex-specific divergence, females typically diverged in a similar direction to males, but to a lesser degree. It is possible that divergence is primarily driven by stronger selection on males, but with expression in females diverging due to correlated responses rather than similar sex-specific selection pressures. In this study, we further hypothesized that the correlated responses in females are due to stronger pleiotropic constraints and aimed to explore the potential for sex-specific population divergence in gene expression to be genetically constrained by $r_{MF}$ and $\tau$.

Here, we assembled an extensive sex-specific gene expression dataset (11 631 genes), which includes (i) a panel of 43 wild-derived inbred lines of *D. serrata* originating from a single natural population in Queensland, Australia, (ii) a sex-specific gene expression atlas consisting of nine tissues, and (iii) a dataset for sex-specific gene expression among eight wild populations spanning the east coast of Australia. With these datasets, we address several questions regarding potential constraints to the evolution of sex-biased gene expression and the faster evolution of male-biased gene expression. First, using the panel of 43 inbred lines, we tested whether male-biased genes have greater genetic variance than female-biased genes. Second, we tested whether sexual dimorphism in male-biased genes was more weakly associated with within- (tissue specificity, $\tau$) and between-sex pleiotropy ($r_{MF}$) than female-biased genes. Third, using data from eight natural populations, we investigated whether the degree of population divergence

in sex-biased expression was similarly associated with within- and between-sex pleiotropy.

## 2. Material and methods

### (a) Custom NimbleGen 135 K microarray

A custom microarray was used to assay gene expression of males and females; the design of the microarray has been previously described [40]. Briefly, five probes per gene (mean of 4.99) were successfully designed for 11 631 expressed sequence tags (ESTs), and each probe was replicated twice, giving a total of 116 174 experimental probes. The EST set used to design the microarray probes was constructed from a combination of Sanger- [41] and Illumina RNA-Seq-derived ESTs. The EST sequences used for microarray design (length $\geq$ 200 bp, $n =$ 11 383) are available in the Genbank Transcriptome Shotgun Archive (TSA) (GAHN00000000.1 at SRA070539) and are a larger set than those originally reported for *D. serrata* [41]. 283 ESTs were shorter than the 200 bp minimum requirement of the TSA and therefore could not be deposited; these have been deposited as electronic supplementary material. The chromosomal location of genes on this microarray has also previously been established [40].

### (b) Biological samples, RNA extraction and microarray hybridization

Gene expression was measured for three sets of flies. First, a panel of 43 wild-derived inbred lines of *D. serrata* were sampled from a single population (Brisbane, Queensland, Australia) and were used to measure male and female gene expression (whole-body), genetic variance for gene expression and the between-sex genetic correlation ($r_{MF}$). These lines, hereafter referred to as the BrisILs, were established by 17 generations of full-sib mating. Second, as previously outlined [40], a laboratory stock from the same location was used to produce a sex-specific gene expression atlas consisting of several body parts: head ($n =$ 4 per sex), thorax (female $n = 3$; male $n = 4$), gonadectomized abdomen ($n = 4$ per sex), ovaries ($n = 3$), testes ($n = 4$) and accessory glands ($n = 4$). The expression atlas was used to assess whether genes had sex-limited expression (expressed above background in only one sex), so as to exclude them from further analysis as they are technically not sex-biased, and to measure tissue specificity ($\tau$, see below). Third, as previously described [39], sex-specific gene expression of eight wild populations was measured to assess potential constraints to population-specific divergence in sex-biased expression.

All flies were reared in 50 ml vials containing standard yeast medium and maintained at 25°C with a 12 L : 12 D cycle. Offspring were reared across nine replicate vials that were density controlled by mating three males and females, collected as virgins with the use of light $CO_2$ anaesthesia and held for 3 days in same-sex groups of five flies. After this time, two replicate pools of 30 flies per line/population per sex and four replicate pools of 100 flies for each tissue sample were snap-frozen in liquid nitrogen without the use of $CO_2$ anaesthesia; each replicate pool originated from a random sample of three rearing vials. RNA extractions were performed by using the Trizol® procedure followed by RNA isolation using RNeasy minikits®. cDNA synthesis, labelling, hybridization and microarray scanning were performed by the Center for Genomics and Bioinformatics, Bloomington, Indiana, USA. Quality control of the array data was performed via the BioConductor 'oligo package' using probe-level models [42–44] and the experimental metrics report provided by NimbleGen. Quality control reduced the BrisILs dataset from $n = 168$ to $n = 142$ hybridizations. All tissue-specific samples ($n = 34$) passed quality control. The expression data for the inbred line and tissue dissections can be found at the NCBI Gene Expression Omnibus under GSE45801. From the population divergence dataset, one male population sample from Cooktown was excluded owing to either a labelling error or female contamination, which reduced this dataset from $n = 48$ to $n = 47$ hybridizations. Expression data for the populations can be found under GSE90733.

### (c) Preprocessing

Common multi-array normalization methods such as robust multi-array normalization (RMA) were not used in this study owing to extensive sex bias, which violates the assumption that most genes are not differentially expressed between samples [45]. Instead, expression measurements were $\log_2$ transformed [44] followed by the use of a mixed linear model-based approach to normalization [46–48]. Model-based approaches account for both biological and non-biological sources of variation simultaneously and were found to produce unbiased results when the above assumption is violated [46]. Outlier probes within each sex were identified via Tukey's criteria (*t*-test *p*-value $<$ 0.0005) [44] and omitted. Of the 16 496 708 measures of expression at the probe level, 1.45% (239 050 probes) were identified as outliers. If only one of the two replicate probes within an array was an outlier, the non-outlier replicate was retained.

### (d) Identification of sex-limited genes

It is possible for sex-limited genes (those likely only expressed in one sex) to be defined based on a measure of tissue specificity [49,50]. For example, genes that are highly specific to a sex-limited tissue such as the testes or ovaries can be classified as male- or female-limited genes, respectively. However, with this method, it is possible that some sex-limited genes will be misclassified if they are, for example, broadly expressed across tissues in one sex but not expressed in the other sex. Also, if a sex-limited gene is expressed at a low level in one or more tissues of a single sex, then it may not reach the tissue-specificity threshold. For these reasons, we calculated an expression threshold as the mean plus two standard deviations of 2 840 000 random probes (20 000 per array) (mean raw fluorescence = 120.8257, s.d. = 136.8326) [51]. Using this threshold, genes were classified as sex-limited if they were not expressed in any tissue of one sex but were expressed in at least one tissue of the opposite sex. A total of 177 genes were classified as female-limited using this method and 1435 as male-limited. Most sex-limited genes were highly specific to the gonads, as previously reported [40].

### (e) Gene expression tissue specificity ($\tau$)

Tissue specificity has been used as a proxy for pleiotropy based on the assumption that genes expressed in a large number of tissues are more likely to affect multiple traits than genes expressed in small number of tissues [14,52]. Using the *D. serrata* sex-specific gene expression atlas [40], we quantified tissue specificity as

$$\tau = \frac{\sum_{i=1}^{n} 1 - E_i/E_{\max}}{n-1}, \tag{2.1}$$

where $E_i$ is the mean expression of tissue $i$ and $E_{\max}$ is the maximum tissue-specific mean expression across all tissues in both sexes; $n$ is the total number of tissue samples in the atlas [30]. In our case, $n = 9$ as it includes male- and female-specific samples for each of the three tissues head, thorax and abdomen plus the three sex-limited tissues of ovary, testes and accessory gland. For example, if a gene was highly expressed in the testes or ovaries but lowly expressed in all other tissues, it had a high value of $\tau$ (greater than 0.95). Similarly, for a gene that

was highly expressed in the gonads of both sexes or head of both sexes, $\tau$ was greater than 0.9. By contrast, a gene that had similar expression across several tissues such as the abdomen and gonads of both sexes had moderate $\tau$ values (less than 0.75), and genes that were expressed at a similar level in all tissues had low $\tau$ values (less than 0.5).

### (f) Sex bias, genetic variance and the between-sex genetic correlation ($r_{MF}$)

The following gene-specific bivariate mixed effects model was fitted to the BrisIL dataset and used to simultaneously estimate sex bias, male and female genetic variances, and the between-sex genetic correlation ($r_{MF}$):

$$\mathbf{Y} = \mu + \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\boldsymbol{\theta}_{\mathbf{G}} + \boldsymbol{\epsilon}, \tag{2.2}$$

where $\mathbf{Y}$ is a stacked column vector of expression observations for males and females, $\mathbf{X}$ is a design matrix linking the fixed effects microarray processing *batch* and *sex* to the fixed effect estimates in $\boldsymbol{\beta}$, and $\mathbf{Z}$ is a design matrix linking *lines* to a vector of unknown random effects $\theta_{\mathbf{G}}$. We assumed that the *line* effects followed a multivariate normal distribution with $\mathbf{Z} \sim N(0, \mathbf{G} \otimes \mathbf{Z})$ where $\mathbf{G}$ is the genetic variance–covariance matrix, $\mathbf{G} = \begin{bmatrix} \sigma^2_{\mathrm{M}} & \sigma^2_{\mathrm{MF}} \\ \sigma^2_{\mathrm{MF}} & \sigma^2_{\mathrm{F}} \end{bmatrix}$ and $\otimes$ denotes the Kronecker product. The residual errors were also assumed to be normally distributed such that $\boldsymbol{\epsilon} \sim N(0, \boldsymbol{\Sigma} \otimes \mathbf{I})$, where $\boldsymbol{\Sigma} = \begin{bmatrix} \sigma^2_{\varepsilon\mathrm{M}} & 0 \\ 0 & \sigma^2_{\varepsilon\mathrm{F}} \end{bmatrix}$ and $\mathbf{I}$ is the identity matrix. Note the exclusion of a residual level between-sex covariance term which cannot be estimated because no individuals are simultaneously male and female. Although the between-sex genetic correlation can be estimated as $r_{\mathrm{MF}} = \sigma^2_{\mathrm{MF}} / \sqrt{\sigma^2_{\mathrm{M}} \times \sigma^2_{\mathrm{F}}}$ using the individual elements of $\mathbf{G}$, we estimated the correlations directly using the TYPE = UNR option in Proc MIXED. This was done to simplify hypothesis testing using likelihood ratio tests. The SAS code for fitting model (2.2) is supplied in the electronic supplementary material.

Genetic variances were tested for a difference from zero using likelihood ratio tests (LRTs) [53]. Likewise, LRTs were used to test $r_{\mathrm{MF}}$ for a difference from zero. A false discovery rate threshold was set at 5% [54]. Sex-specific broad-sense heritabilities were calculated as the proportion of the total within-sex phenotypic variance ($V_{\mathrm{P}}$) that was attributable to the sex-specific genetic variance (for example in males, $V_{\mathrm{G(M)}}/V_{\mathrm{P(M)}}$, where $V_{\mathrm{P(M)}} = V_{\mathrm{line(M)}} + V_{\mathrm{E(M)}}$) [20].

Sex bias in gene expression was measured as mean $\log_2$ male − mean $\log_2$ female expression, where mean male and female expression were estimated from the fixed effect of *sex* in model (2.2) using the DIFF option of the LSMEANS statement in PROC MIXED from SAS v. 9.3 [53]. It has been pointed out by Stewart *et al*. [55] that in high-powered experiments such as this, many genes classified as sex-biased based on statistical tests alone may be misleading and biologically irrelevant. For this reason, we only classified genes as sex-biased if, in addition to being significantly different in mean expression level between the sexes (FDR < 5%), there was an at least twofold expression difference between the sexes [14].

### (g) Correlations between sex bias and pleiotropy metrics

Negative relationships between $r_{\mathrm{MF}}$ and absolute sex bias within a population or changes in sex bias among populations are consistent with evolutionary constraints due to between-sex pleiotropy [15,27,56]. Similarly, positive associations between $\tau$ and sex bias within or changes in sex bias among populations are consistent with pleiotropic constraints [14]. These associations and their dependence on the direction of sex bias (male- versus female-biased) were assessed statistically using permutation-based analysis of covariance models (ANCOVA), which importantly are not reliant on the assumption of Gaussian distributed residuals [57]:

$$abs(sexbias) = sex\_bias\_direction + \tau + sex\_bias\_direction \times \tau + error \tag{2.3}$$

$$abs(sexbias) = sex\_bias\_direction + r_{\mathrm{MF}} + sex\_bias\_direction \times r_{\mathrm{MF}} + error, \tag{2.4}$$

where *sex_bias_direction* is a categorical coding variable indicating whether a gene was male- or female-biased. Significant interactions in these models indicate differences in strength and/or sign of association between male- and female-biased genes. Models (2.3) and (2.4) were fitted using the *aovperm*() function implemented in the R/permuco package [58]. Spearman's rank correlations were also reported for relationships within each sex bias class.

### (h) Population divergence in sex-biased gene expression

Population divergence in gene expression has been previously assessed [39]. Briefly, the following fixed effects model was used to assess divergence in gene expression between populations:

$$expression = sex + pop + sex \times pop + error. \tag{2.5}$$

Expression of each gene was standardized to a mean of zero and unit variance [$\sim N(0, 1)$] across the entire dataset so that divergence level could be compared between genes. In this model, the *sex* × *pop* interaction was used to test whether any divergence among populations was sex-specific. It was assessed via Type III tests [59] and multiple test corrected to a false discovery rate of 5% [54].

To assess constraints on sex differences in population divergence, we first estimated the magnitude of divergence using a local effect size known as Cohen's $f^2$ for the *sex* × *pop* interaction from model (2.5):

$$f^2 = \frac{R^2_{AB} - R^2_A}{1 - R^2_{AB}}, \tag{2.6}$$

where $B$ is the fixed effect of interest (*sex* × *pop*), $A$ represents all other variables, $R^2_{AB}$ is the proportion of variance accounted for by $A$ and $B$ together, and $R^2_A$ is the proportion of variance accounted for by $A$. Therefore, the numerator of equation (2.6) is the proportion of variance accounted for by the *sex* × *pop* interaction, beyond all other factors [60]. We then refitted models (2.3) and (2.4) using Cohen's $f^2$ as the response variable to assess relationships between population divergence and $\tau$ and $r_{\mathrm{MF}}$.

## 3. Results

### (a) Sex-limited and sex-biased gene expression

We first used the gene expression atlas to detect sex-limited genes. Of the 11 631 genes (ESTs) present on the microarray platform, 1612 genes were classified as sex-limited. These genes were those expressed above the expression threshold (defined by random probes) in at least one tissue of one sex while failing to exceed the threshold for all tissues in the opposite sex. The overwhelming majority of sex-limited genes were male-limited (1435 genes) as opposed to female-limited (177 genes). Most expressed genes were expressed in both sexes (8331). We removed sex-limited genes from

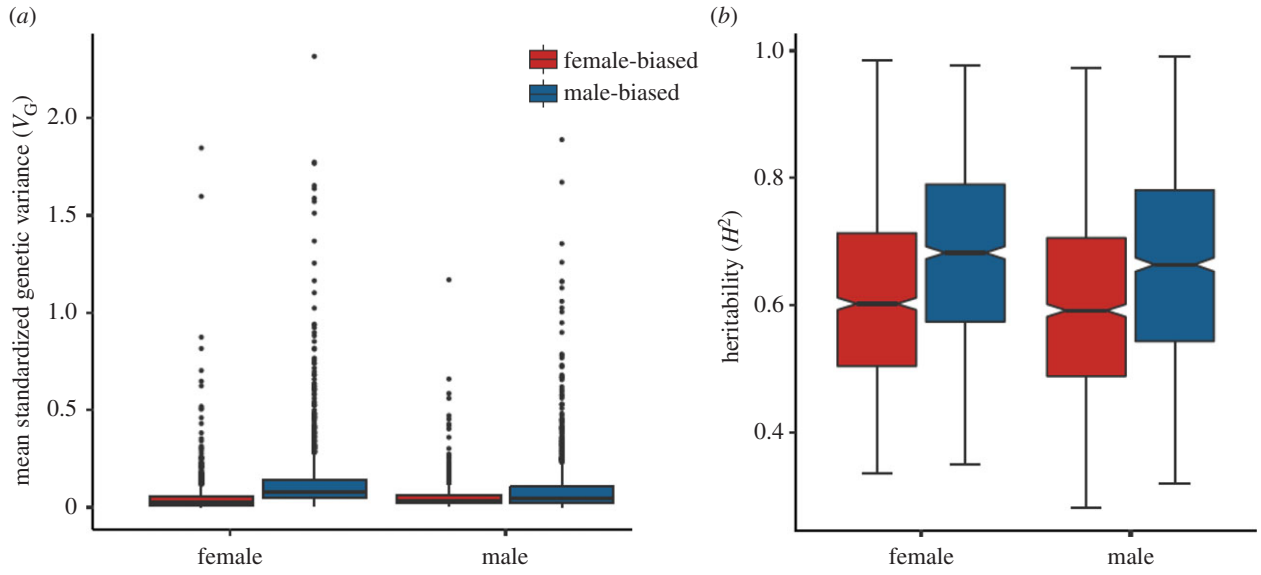


**Figure 1.** Boxplots showing the distribution of genetic variance (*a*) and broad-sense heritability estimates (*b*) for 2369 co-expressed sex-biased genes in the Brisbane population. (Online version in colour.)

further analyses of the 43 inbred lines from Brisbane (BrisILs). In this dataset, there were more female-biased (2450) than male-biased genes (2004). While most studies of other *Drosophila* species report more male- than female-biased genes [61], the large excess of male-biased genes is due to their inclusion of male-limited genes in this category. We found similar results previously among the eight natural populations of *D. serrata* [39].

## (b) Broad-sense heritability ($H^2$) of gene expression

Transcript abundance of most co-expressed genes was heritable, that is, the among-line variance component of model (2.2) was significantly greater than zero. A total of 6147 genes (73.8%) were heritable in males, and 6151 (73.8%) were heritable in females. The intersection of these two sets led to a total of 4927 (59.1%) co-expressed genes that were heritable in both sexes. As our goal was to compare the properties of male- and female-biased genes, we focused our further analyses of genetic constraint on the set of 2369 co-expressed genes that were sex-biased and heritable in both sexes.

## (c) Do male-biased genes have higher evolvability?

One possible reason for an elevated rate of male-biased gene expression evolution is that these genes have greater evolutionary potential in the form of genetic variance. We found this to be the case across the 2369 co-expressed sex-biased genes with non-zero heritability that were analysed. In the panel of inbred lines, male-biased genes had on average higher (mean standardized) genetic variance than female-biased genes regardless of whether gene expression was measured in males (figure 1*a*; $F_{1,2367} = 88.582$, $p = 1.12 \times 10^{-20}$) or females ($F_{1,2367} = 186.31$, $p = 6.89 \times 10^{-41}$). This result was also robust to scale of measurement. When genetic variation was compared between male- and female-biased genes on a broad-sense heritability scale, a similar elevation was found for male-biased genes (figure 1*b*; males: $F_{1,2367} = 102.09$, $p = 1.58 \times 10^{-23}$; females: $F_{1,2367} = 121.71$, $p = 1.24 \times 10^{-27}$).

## (d) Correlation between tissue specificity ($\tau$) and sex bias

A positive correlation between $\tau$ and sex bias has been used to infer that pleiotropy across tissues constrains the evolution of sexually dimorphic expression [14,22,34]. However, it is thus far unknown whether this correlation varies according to the direction of sex bias. A weaker association for male-biased genes may help to explain their faster evolution. Although mean $\tau$ was very similar for male- (0.6679) and female-biased genes (0.6677), there was significant interaction between sex bias direction and, $\tau$, suggesting a difference in the correlation between $\tau$ and sex bias for male- and female-biased genes ($F_{1,2365} = 57.05$, permutation $p = 1.00 \times 10^{-4}$; figure 2). For both categories, there was a positive correlation between sex bias and $\tau$. However, the correlation was weaker for male-biased genes (Spearman's $\rho = 0.31$, $p = 1.1 \times 10^{-27,}$ $n = 1165$) than female-biased genes (Spearman's $\rho = 0.58$, $p = 9.7 \times 10^{-109}$, $n = 1204$). One issue with the calculation of the metrics is that the inclusion of sex-limited tissues (accessory glands, testes and ovaries) may upwardly bias the positive relationships between $\tau$ and sex bias [18]. In an attempt to remove this possible source of bias, we recalculated $\tau$ after omitting the sex-limited tissues. Upon reanalysis, the significant interaction and positive correlations remained ($\tau \times$ *sex_bias_direction*: $F_{1,2365} = 92.86$, permutation $p = 0.0001$), as did the weaker correlation for male-biased genes relative to female-biased genes (male-biased, Spearman's $\rho = 0.17$, $p = 4.9 \times 10^{-09}$, $n = 1165$; female-biased, Spearman's $\rho = 0.41$, $p = 1.7 \times 10^{-49}$, $n = 1204$). Combined, these results suggest that the evolution of sex bias may be less constrained by within-sex pleiotropy for male-biased genes than it is for female-biased genes.

## (e) Correlations between $r_{MF}$ and sex bias

The $r_{MF}$ estimates for co-expressed genes with heritable expression in the *D. serrata* BrisILs had a mean of 0.44, median of 0.46 and standard deviation of 0.31 (figure 3). These overall values of $r_{MF}$ for gene expression are very similar to those reported in *D. melanogaster*, estimated using

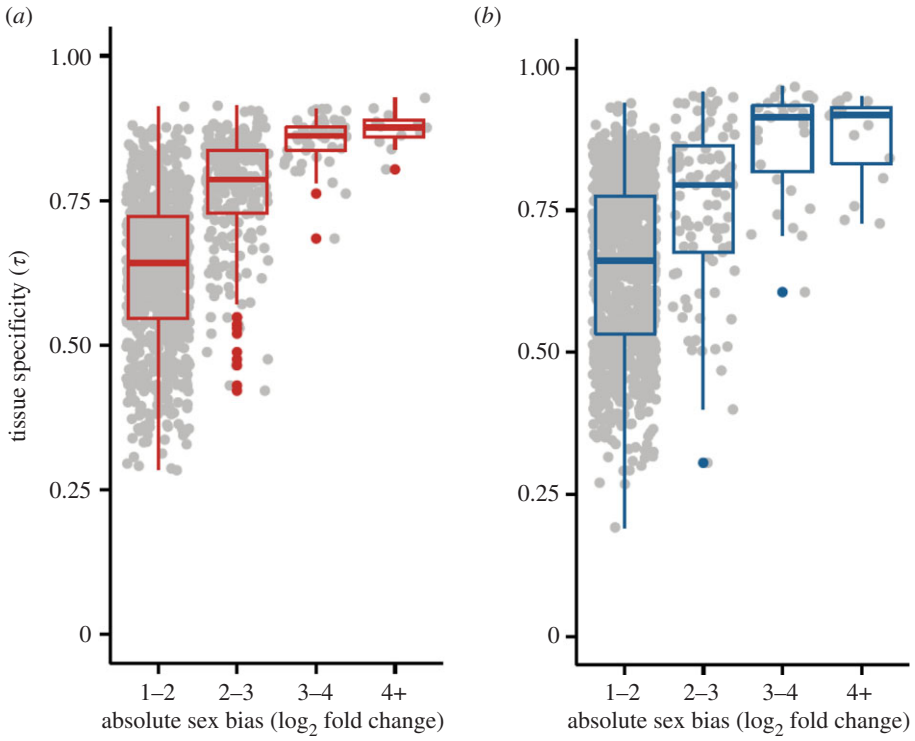


**Figure 2.** Association between tissue specificity ($\tau_{MF}$) and sex bias for female-biased (*a*) and male-biased genes (*b*) in *D. serrata*. Grey points are the estimates of $\tau_{MF}$ for each gene and their level of sex bias within the Brisbane population. Overall, tissue specificity ($\tau_{MF}$) was strongly correlated with absolute sex bias. However, the correlation between $\tau_{MF}$ and sex bias was weaker for male-biased than female-biased genes (see Results). Boxplots are added to enhance visual display only and do not indicate statistical analyses. (Online version in colour.)

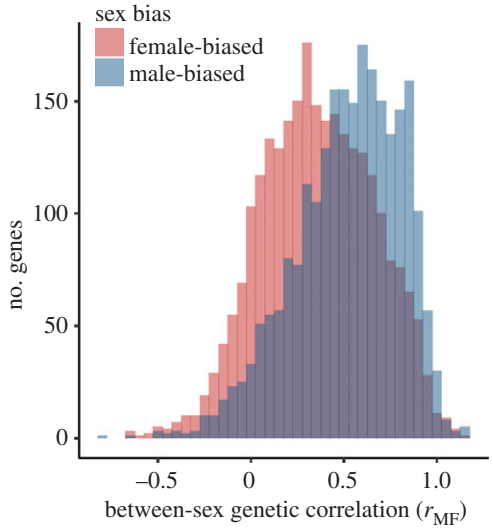


**Figure 3.** Distribution of between-sex genetic correlation ($r_{MF}$) estimates for 2369 genes that had heritable expression levels and were expressed in both sexes (co-expressed genes). (Online version in colour.)

similar breeding designs [15,62]. There was a significant interaction between sex bias direction and $r_{MF}$ ($F_{1,2365} = 25.99$, permutation $p = 0.0001$; figure 4) which was driven by a moderate negative correlation between $r_{MF}$ and sex bias for male-biased genes (Spearman's $\rho = -0.12$, $n = 1\,165$, $p = 3.736 \times 10^{-5}$); and a negative but far weaker correlation for female-biased genes, which could not be distinguished from zero (Spearman's $\rho = -0.008$, $n = 1\,204$, $p = 0.7663$). In terms of constraints, these results suggest that, relative to female-biased genes, the evolution of sexual dimorphism in male-biased genes may be more tightly influenced by between-sex pleiotropy.

One possible explanation for the generally stronger associations we saw for male-biased genes and $r_{MF}$ is that perhaps male-biased genes were, on average, more dimorphic than female-biased genes. If this were the case, genetic constraints on the evolution of sex differences might be more likely. We tested this by comparing the absolute sex bias between male- and female-biased genes and found the opposite pattern. Sex bias for female-biased genes was higher than that of male-biased genes (Wilcoxon test, $W = 550\,680$, $p < 2.2 \times 10^{-16}$, median female bias = 1.55, median male bias = 1.37), suggesting this is unlikely to account for the increased negative association.

## (f) Pleiotropy and sex differences in population divergence

We previously found that adult gene expression in *D. serrata* exhibits extensive divergence between natural populations [39]. Although sex differences in population divergence were apparent, with much of the divergence occurring in males and for genes with male-biased expression, often divergence in these genes was positively correlated between males and females [39]. This pattern suggests that pleiotropy between sexes may have biased divergence between populations. To investigate this further, here we examined how our measures of both tissue specificity ($\tau$) and the between-sex genetic correlation for gene expression ($r_{MF}$) covaried with the degree of sex-dependent population divergence, measured via Cohen's $f^2$.

Tissue specificity, $\tau$, was significantly associated with sex-dependent population divergence for both male- and female-biased genes ($\tau$: $F_{1,2365} = 15.93$, permutation $p = 0.0001$; figure 5), but there was no interaction with sex bias

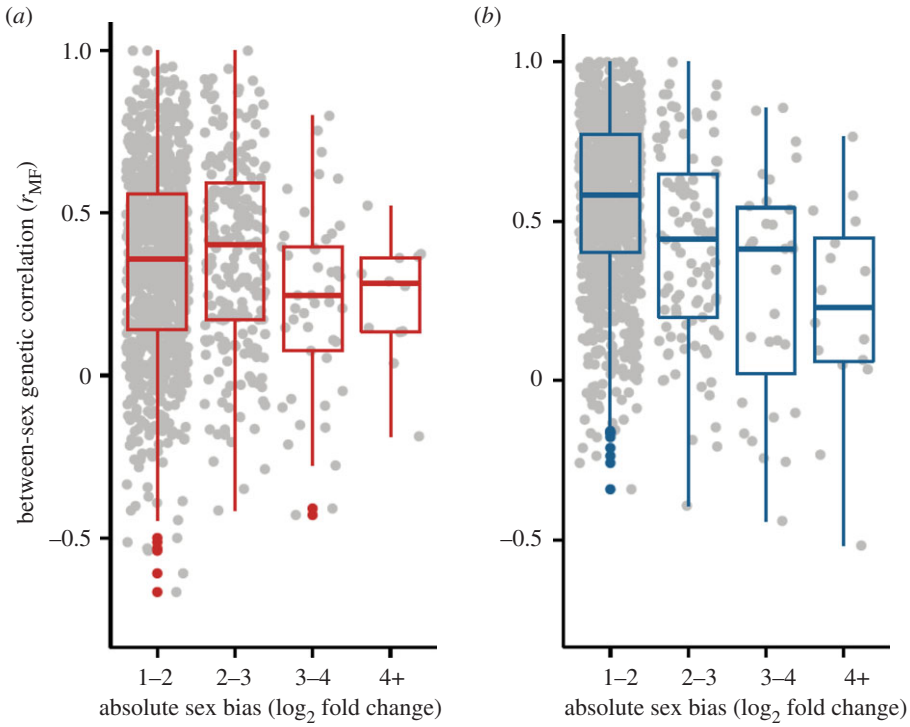


**Figure 4.** Within-population correlation between $r_{MF}$ and sex bias for female-biased (*a*) and male-biased (*b*) genes. Grey points are restricted maximum likelihood (REML) estimates of $r_{MF}$ for each gene measured in the Brisbane population and their corresponding level of sex bias in the population. Boxplots are added to enhance visual display only and do not indicate statistical analyses. (Online version in colour.)

direction ($\tau \times$ *sex_bias_direction*: $F_{1,2365} = 1.07$, permutation $p = 0.3035$). The association was positive and moderate for both male- and female-biased genes (figure 5) (male-biased Spearman's $\rho = 0.11$, $n = 1{,}165$, $p = 0.0001$; female-biased Spearman's $\rho = 0.14$, $n = 1{,}204$, $p = 2.248 \times 10^{-6}$). Using the more conservative test where $\tau$ was measured with sex-limited tissues excluded (see above), there was a marginally non-significant interaction ($\tau \times$ *sex_bias_direction*: $F_{1,2365} = 3.24$, permutation $p = 0.0761$) where divergence in male-biased genes was not associated with $\tau$ (male-biased Spearman's $\rho = 0.05$, $n = 1{,}165$, $p = 0.1200$), but there was a significant positive association for female-biased genes (Spearman's $\rho = 0.07$, $n = 1{,}204$, $p = 0.0127$).

Similar to the pattern seen for within-population sexual dimorphism, there was also a significant interaction between $r_{MF}$ and sex bias direction for sex-dependent divergence among populations ($F_{1,2365} = 25.99$, permutation $p = 0.0001$; figure 6). This interaction was due to a much stronger negative association between $r_{MF}$ and Cohen's $f^2$ for male-biased genes (Spearman's $\rho = -0.21$, $n = 1{,}165$, $p = 7.33 \times 10^{-13}$) than female-biased genes (Spearman's $\rho = -0.08$, $n = 1{,}204$, $p = 0.0055$).

## 4. Discussion

An intriguing observation related to the evolution of sex-biased gene expression is that, for many species, male-biased genes tend to evolve at a rapid rate compared with female-biased genes across several species [1,2,61,63–68]. Although this may be caused by stronger selection on males, the potential roles of weaker evolutionary constraints and/or greater genetic variance remain comparatively unexplored. Our goal in this study was to address these latter aspects of genetic architecture on both within- and among-population scales.

### (a) Male-biased genes have higher evolvability

Because the potential evolutionary response to selection is proportional to the strength of selection and the magnitude of heritability [19], the evolvability of a trait is proportional to the additive genetic variance [69,70]. Therefore, if the strength of selection was equal on male-biased and female-biased genes, but there was more genetic variance for male-biased genes, male-biased genes could respond faster and subsequently evolve faster. Note that greater genetic variance only indicates that male-biased genes have more evolutionary potential and is not indicative of an adaptive explanation. We found this to be the case in *D. serrata*, where genetic variance in gene expression for male-biased genes was, on average, greater than for female-biased genes. Interestingly, male gene expression did not have higher genetic variance than female gene expression. Such a pattern would be consistent with higher evolvability of male than female gene expression [21]. Instead, across all co-expressed genes analysed, genetic variance and heritability estimates tended to be slightly higher in females than males (figure 1). It is not known whether $V_g$ being greater for male-biased genes than female-biased genes will be replicated in other species. However, it can be seen in the electronic supplementary material of a similar-sized experiment on *D. melanogaster* [62] that (broad-sense) heritability of male-biased genes was similar to that of female-biased genes. Unfortunately, similar heritability does not necessarily imply similar genetic variance and so these data will need reanalysis to permit reliable comparison.

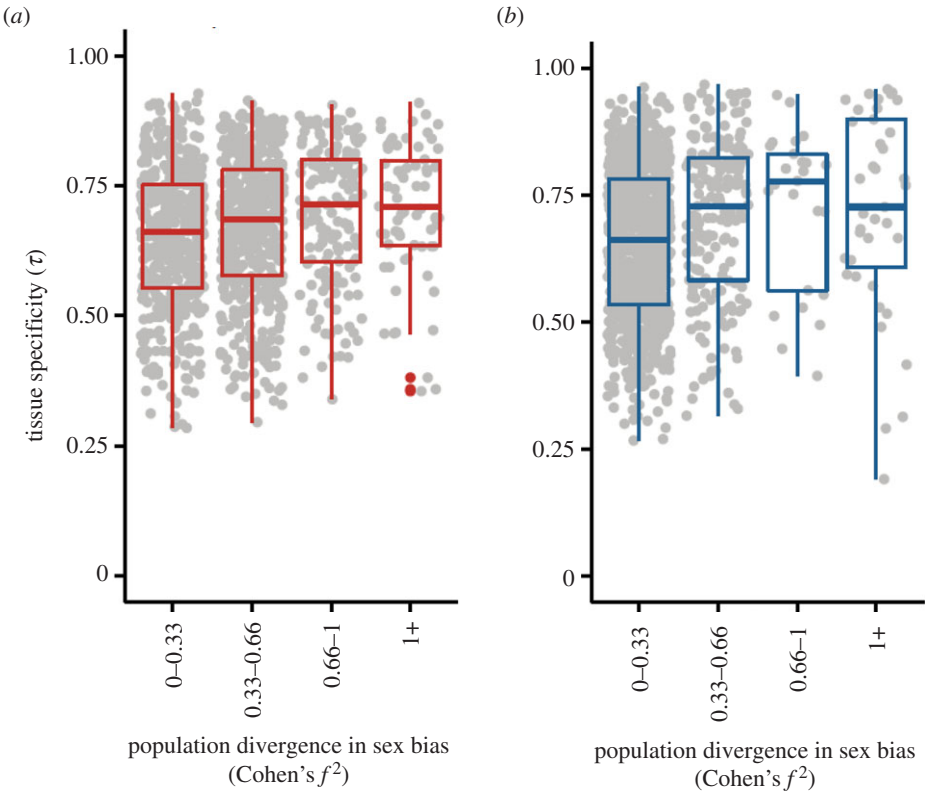


**Figure 5.** The correlation between tissue specificity ($\tau$) and sex-specific divergence in expression level among eight natural populations of *D. serrata*. Grey points are the estimates of $\tau_{MF}$ for each gene and their corresponding values of Cohen's $f^2$. The red boxplots are the distribution of sex-specific divergence for female-biased genes (*a*) and the blue boxplots for male-biased genes (*b*). Boxplots are added to enhance visual display only and do not indicate statistical analyses. (Online version in colour.)

## (b) Pleiotropy and within-population sexual dimorphism

Pleiotropy adds complexity to the response to selection because a mutation could be beneficial for one trait but at the same time detrimental to another [26–29]. It has previously been shown in mice (*M. musculus*), chickens (*G. gallus*) [14] and flies (*D. melanogaster* and *D. pseudoobscura*) [18,22] that sex-biased genes tend to be less pleiotropic than unbiased genes, leading to the suggestion that pleiotropy might constrain the evolution of sex-biased gene expression. Therefore, one possible explanation for the rapid evolution of male-biased genes is that they are less constrained by pleiotropy. We found this to be the case in *D. serrata*. Although there was practically no difference in the magnitude of pleiotropy as measured by tissue specificity ($\tau$), we found that the correlation between $\tau$ and sex bias was considerably weaker for male-biased genes than it was for female-biased genes, a pattern consistent with male-biased genes being less constrained by pleiotropy than female-biased genes. This result contrasts with that observed in *D. melanogaster* [18], where $\tau$ was considerably greater for male-biased genes than female-biased genes, as was the correlation between $\tau$ and sex bias for male-biased genes. However, this result was likely due to the inclusion of sex-limited genes as sex-biased, which would have upwardly biased $\tau$ and the correlation for male-biased genes. When gonadectomized flies were compared by Meisel [18], the results were comparable to our own, likely due to the exclusion of many sex-limited genes when sex-limited tissues were excluded.

In species with two sexes, pleiotropy between sexes could constrain the evolution of gene expression. Although results from studies examining $r_{MF}$ as a constraint to higher order sexually dimorphic phenotypes such as morphology or behavioural traits have been mixed [33,71–77], this may be due to the lower power afforded by the use of relatively few traits in each species [32]. By contrast, gene expression studies allow $r_{MF}$ to be associated with sex bias for thousands of traits in a single species. Indeed, a highly statistically significant negative association was detected between $r_{MF}$ and sex bias in *D. melanogaster* [15]. Our goal here was to assess whether such associations might be weaker for male-biased genes, which tend to diverge in expression level faster and to a greater extent in *Drosophila* than female-biased genes.

Interestingly, $r_{MF}$ was significantly lower for female-biased genes than male-biased genes (figure 4) (Mann–Whitney test: $W_{(2448,2283)} = 2\,099\,300$, $p = 1.3 \times 10^{-81}$), although it is unclear why this is the case. It is expected that genes measured with poor precision will bias the estimate of $r_{MF}$ downwards and, assuming that the proportion of total variance explained by genetic variance (heritability) is a reasonable approximation for precision, this appeared to be the case in *D. melanogaster*, where heritability was indeed positively correlated with $r_{MF}$ [15]. We too found a positive correlation between average heritability across the sexes and $r_{MF}$ for both female-biased (Spearman's $\rho = 0.25$, $p = 2.3 \times 10^{-37}$) and male-biased genes (Spearman's $\rho = 0.33$, $p = 5.1 \times 10^{-58}$), and found that heritability was, on average, slightly lower for female-biased genes (60%) relative to male-biased genes (66%) (Mann–Whitney test: $W_{(2448,2283)} = 1\,896\,100$, $p = 1.3 \times 10^{-49}$). It is therefore possible that $r_{MF}$ for female-biased genes appears lower simply because measurements of expression were less precise than for male-biased genes. Alternatively, the spectrum of pleiotropic

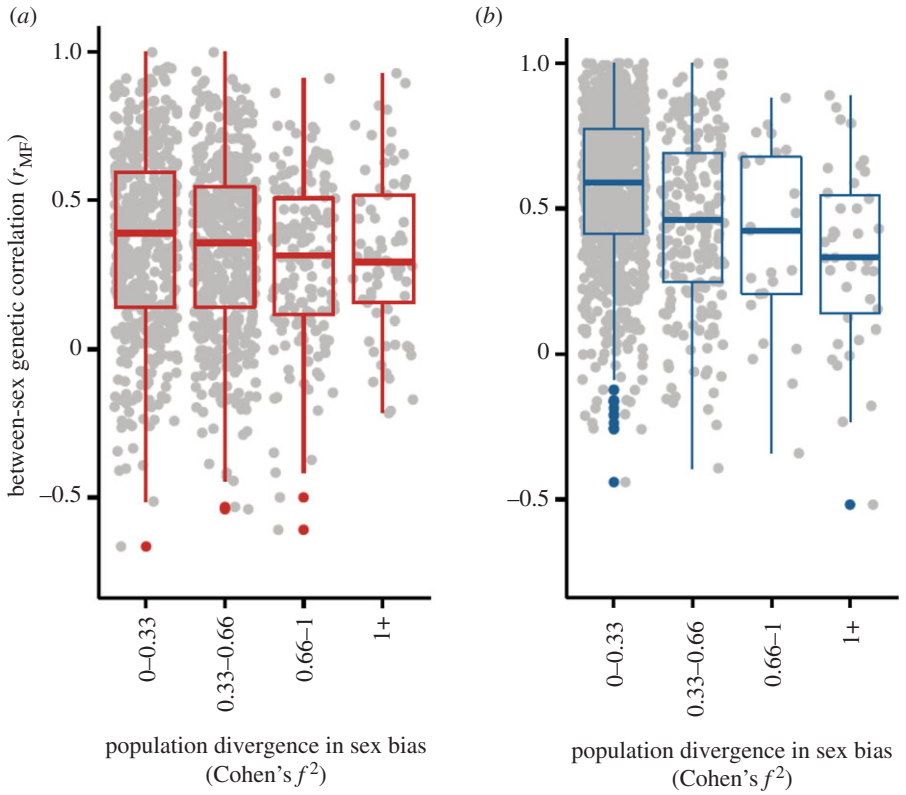


**Figure 6.** Negative correlation between $r_{MF}$ and sex-specific divergence in expression level among eight natural populations of *D. serrata*. Grey points are the REML estimates of $r_{MF}$ for each gene measured in the Brisbane population and their corresponding values of Cohen's $f^2$. The red boxplots are the distribution of sex-specific divergence for female-biased genes (*a*) and the blue boxplots are for male-biased genes (*b*). Boxplots are added to enhance visual display only and do not indicate statistical analyses. (Online version in colour.)

mutations might differ between male- and female-biased genes, but this remains untested. Further, if sexually antagonistic selection persists for a long period of time, $r_{MF}$ may eventually decline [56]. If sexually antagonistic selection on male-biased genes was driven through selection on males (e.g. via sexual selection) and was more variable in time and space [78,79], there might not be sufficient time for a breakdown of $r_{MF}$ to occur. Interestingly, lower $r_{MF}$ for female-biased genes may help explain our finding of their weaker association with sex bias. While male-biased genes were quite strongly negatively associated with $r_{MF}$, the evidence for between-sex pleiotropy constraining female-biased genes was tentative at best (figure 4). Given that male-biased genes are likely more important for male fitness than female fitness [80], our results suggest that the evolution of sexual dimorphic expression through selection on males might more commonly be constrained by between- as opposed to within-sex pleiotropy.

## (c) Pleiotropy and sex differences in population divergence

Gene expression divergence was extensive among eight natural populations of *D. serrata*, with the vast majority of divergence occurring for male-biased genes [39]. Furthermore, divergence was typically of a greater magnitude in males than females. Such sex-specific divergence ultimately requires population-specific changes in sex bias. We hypothesized that females were diverging in a similar manner to males, albeit less so, due primarily to constraints between sexes. For example, if $r_{MF}$ for a gene is high, then sex-dependent evolution will be limited regardless of the strength of sex-specific selection [27,31]. In this study, we found that both forms of pleiotropy tested ($\tau$ and $r_{MF}$) had associations consistent with constraints on the sex-dependent divergence of gene expression. In particular, similar to the within-population analyses, the correlation between divergence and $r_{MF}$ was stronger for male- than female-biased genes. This provides some support for the hypothesis that expression divergence in *D. serrata* might be largely driven by selection on males with female divergence in many cases occurring via correlated rather than similar sex-specific selection pressures [39]. In other words, our results suggest that *D. serrata* populations diverge in mean phenotype and sexual dimorphism largely in response to local differences in selection on males (reflecting either male-specific viability adaptations, or patterns of sexual selection shaped by local environments) and that, at the genetic level, much of this divergence entails changes in expression of male-biased genes. The evolution of male-biased genes could therefore represent a key mechanism of population divergence and local adaptation.

## (d) Conclusion and caveats

Our quantitative genetic analyses of sex-biased gene expression in *D. serrata* support the ideas that the rapid evolution of male-biased gene expression could be explained by their higher genetic variance and/or narrower expression patterns ($\tau$). However, our findings are not consistent with the idea of weaker constraint from between-sex pleiotropy on male-biased genes. Instead, $r_{MF}$ was strongly negatively associated with sex bias for male-biased genes and its variation among natural populations, suggesting it might be an

important form of constraint when divergence is driven by selection on males [39].

There are some important caveats to our results that should be kept in mind. First, although whole-organism transcription is a high-dimensional phenotype, many of our analyses necessarily assume a degree of independence between genes. Genes exhibit correlated expression patterns and so the true number of genetically independent expression traits available for selection and drift to act upon is likely to be lower than the total number of measured genes [81]. Understanding how this non-independence could influence the patterns we see here will require the future application of sophisticated and computationally intensive multivariate methods. Although some statistical tools appear promising, such as large matrix completion [81] and Bayesian sparse factor analysis [82], their application to studies of sexually co-expressed genes is not straightforward because the inclusion of male and female expression traits for each gene effectively doubles the number of traits to be analysed and no residual covariances between sexes can be estimated. Second, while they are consistent with evolutionary constraints, negative associations between sex bias and measures such as $r_{MF}$ do not by themselves tell us about the processes leading to the evolution of sexual dimorphism. Equally plausible explanations for these associations are that $r_{MF}$ indeed breaks down in the face of persistent sex-specific selection or perhaps more simply genes with initially low $r_{MF}$ can more readily become dimorphic than those with high $r_{MF}$ [34].

Our findings also highlight a number of intriguing questions about adaptation and diversification of sexual lineages. In particular, how is higher genetic variance in male-biased genes generated and maintained? How does within-sex pleiotropy constrain the evolution of sex-biased genes, and why does between-sex pleiotropy seem to have a weaker effect? What are the ecological factors that drive male-biased adaptation, and how do sexual and viability selection contribute to this process? And why does female-specific directional selection appear to play a lesser role than male-biased directional selection in driving population divergence and local adaptation? Is it the case that female expression levels are instead under stronger stabilizing selection? It is becoming increasingly clear that diversification and local adaptation in sexual lineages are shaped to a considerable degree by the interplay of sex-specific selection and sex-specific genetic constraints. Our results suggest that the fitness effects and evolutionary potential of sex-biased genes could be particularly important and represent a key problem in the study of adaptive evolution.

Data accessibility. Gene expression data for this study have been deposited in the Gene Expression Omnibus (BrisILs: GSE45801, Population data: GSE90733). The intermediate data file used to analyse pleiotropy metrics and their associations with sex bias has been uploaded as supplementary material to figshare along with R code. The SAS code to fit the linear model in equation (2.1) has also been uploaded to figshare.

Authors' contributions. S.L.A., R.B. and S.F.C. designed the study and wrote and edited the paper. S.L.A. performed the experiments and analysed the data.

Competing interests. We declare we have no competing interests.

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