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Sex-biased transcriptome divergence along a latitudinal gradient

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Abstract

Sex-dependent gene expression is likely an important genomic mechanism that allows sex-specific adaptation to environmental changes. Among *Drosophila* species, sex-biased genes display remarkably consistent evolutionary patterns; male-biased genes evolve faster than unbiased genes in both coding sequence and expression level, suggesting sex differences in selection through time. However, comparatively little is known of the evolutionary process shaping sex-biased expression within species. Latitudinal clines offer an opportunity to examine how changes in key ecological parameters also influence sex-specific selection and the evolution of sex-biased gene expression. We assayed male and female gene expression in *Drosophila serrata* along a latitudinal gradient in eastern Australia spanning most of its endemic distribution. Analysis of 11 631 genes across eight populations revealed strong sex differences in the frequency, mode and strength of divergence. Divergence was far stronger in males than females and while latitudinal clines were evident in both sexes, male divergence was often population specific, suggesting responses to localized selection pressures that do not covary predictably with latitude. While divergence was enriched for male-biased genes, there was no overrepresentation of X-linked genes in males. By contrast, X-linked divergence was elevated in females, especially for female-biased genes. Many genes that diverged in *D. serrata* have homologs also showing latitudinal divergence in *Drosophila simulans* and *Drosophila melanogaster* on other continents, likely indicating parallel adaptation in these distantly related species. Our results suggest that sex differences in selection play an important role in shaping the evolution of gene expression over macro- and micro-ecological spatial scales.

Keywords: divergence, latitudinal cline, parallel divergence, sex-biased gene expression

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Introduction

A large fraction of the genomes of dioecious species are sex-biased in transcription (Ellegren & Parsch 2007; Parsch & Ellegren 2013; Ingleby *et al.* 2015), with extremes of up to 90% of all genes exhibiting sexually dimorphic expression (e.g. Drosophila melanogaster, Ayroles *et al.* 2009; Innocenti & Morrow 2010; Ranz *et al.* 2003; Zhang *et al.* 2007). The sex-dependent regulation of gene expression is a key genomic mechanism for adaptation with two sexes, where selection is often sex specific (Cox and Calsbeek 2009). Sex-biased gene expression provides a mechanism whereby males and females can escape the pleiotropic constraints of a shared proteome, allowing the sexes to approach divergent fitness optima without coding sequence divergence between them. Indeed, many sexually dimorphic phenotypes are likely underlain by sex differences in gene expression (Williams & Carroll 2009; Loehlin *et al.* 2010).

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At the interspecific level, sex-biased genes show some remarkably consistent evolutionary patterns (Ellegren & Parsch 2007). First, sex-biased (particularly male-biased) genes tend to diverge between species much faster than unbiased genes, both in terms of coding sequence (Zhang *et al.* 2004; Ellegren & Parsch 2007; Assis *et al.* 2012; Parsch & Ellegren 2013) and expression level (Meiklejohn *et al.* 2003; Ellegren & Parsch 2007; Assis *et al.* 2012); however, such observations may be species specific (Metta *et al.* 2006). It has been suggested that excessive divergence in male-biased genes is due to stronger and more variable selection on males (Connallon & Knowles 2005). Consistent with such an adaptive interpretation, the accelerated divergence of sex-biased genes is often accompanied by evidence for positive selection (Meiklejohn *et al.* 2003; Khaitovich *et al.* 2005; Nielsen *et al.* 2005; Zhang & Parsch 2005; Proschel *et al.* 2006). A second pattern seen in interspecific comparisons of sex-biased gene evolution is the 'faster-X effect' (Rice 1984; Charlesworth *et al.* 1987; Betancourt *et al.* 2002; Lu & Wu 2005; Nielsen *et al.* 2005; Meisel & Connallon 2013). Here, while there is a trend towards X-linked male-biased genes, which likely affect male fitness more than female fitness (Connallon & Clark 2011), displaying stronger divergence in DNA sequence (Baines *et al.* 2008; Meisel 2011; Grath & Parsch 2012) than unbiased genes, evidence has been mixed (Meisel & Connallon 2013; Avila *et al.* 2014, 2015). Furthermore, while few studies have examined sex-specific divergence in gene expression, it also appears pronounced in male-biased genes (Llopart 2012; Meisel *et al.* 2012) relative to X-linked female-biased genes.

The fact that these evolutionary patterns are consistent across multiple species and pervade both coding sequence and expression level variation suggests that long-term sex differences in fitness optima are significant factors influencing sex-biased gene evolution (Harrison *et al.* 2015). However, because the majority of these inferences have been drawn from interspecific comparisons, we do not know whether the same processes shape sex-biased expression divergence among populations within species. To date, the comparatively few intraspecific studies of sex-biased expression divergence – focusing on *Drosophila* – have produced mixed results. While relatively more male-biased genes (when expressed in males) diverged between *Drosophila melanogaster* populations (Meiklejohn *et al.* 2003; Hutter *et al.* 2008; Zhao *et al.* 2015), the results were not as clear when examining male-biased genes when they were expressed in females (Muller *et al.* 2011), a suggestion that differences in selection between the sexes may result in sex-specific divergence. Similarly, support for the 'faster-X' evolution of gene expression at the intraspecific level is also mixed (Hutter *et al.* 2008; Zhao *et al.* 2015).

Latitudinal clines have a rich history in evolutionary genetics owing to the powerful inference framework they offer for deducing genetically based responses to spatially varying selection (Haldane 1948; Endler 1977). In *Drosophila,* latitudinal clines have been well documented for allele frequencies (Kolaczkowski *et al.* 2011; Reinhardt *et al.* 2014), life history traits (Schmidt *et al.* 2005; Arthur *et al.* 2008; Schmidt & Paaby 2008) and other quantitative traits (Hoffmann & Weeks 2007), and these patterns are thought to reflect the balance between local adaptation and migration (Adrion *et al.* 2015). Parallel divergence along clines between codistributed species or between comparable clines within species strengthens the inference of adaptation (Endler 1986), and there are now many examples of parallel divergence along latitudinal clines on different continents for traits (Coyne & Beecham 1987; James *et al.* 1995; Azevedo *et al.* 1996, 2002; Huey *et al.* 2000; Zwaan *et al.* 2000; Hallas *et al.* 2002; Arthur *et al.* 2008; van Heerwaarden *et al.* 2012; Matute & Harris 2013) and allele frequencies (Oakeshott *et al.* 1982; Fry *et al.* 2008; Reinhardt *et al.* 2014). Geographical variation has also been used to study the dynamics of spatially varying sex-specific selection (Blanckenhorn *et al.* 2006; Chenoweth *et al.* 2008) where divergence in sexual dimorphism may reflect responses to spatially variable sex-specific selection (Connallon 2015). Understanding the microevolution of sex-biased expression requires understanding the roles of both local/microscale ecological variation and broader ecological patterns, such as clinal variation in climate. To date, *Drosophila* studies that have utilized latitudinal clines to study expression divergence have examined only two populations, usually at cline ends, which precludes strong inference about either form of ecological variation.

Here, we have analysed genetic divergence in male and female gene expression among eight natural populations of *Drosophila serrata* spanning approximately 20° of latitude (~2300 km) and much of the species' natural range. The eastern Australian distribution of *Drosophila serrata* is an appealing model for assessing microevolutionary divergence in sex-biased expression for multiple reasons. First, latitudinal divergence is already established for multiple life history, morphological and behavioural traits [development time (Magiafoglou *et al.* 2002; Sgro & Blows 2003); wing shape (Hoffmann & Shirriffs 2002), chill coma resistance (Hallas *et al.* 2002); body size (Hallas *et al.* 2002); and locomotor activity (Latimer *et al.* 2011)]. Second, there is clear evidence for adaptive divergence along the cline for well-studied traits such as cuticular hydrocarbons, which are subject to both natural and sexual selection (Higgie *et al.* 2000; Chenoweth & Blows 2008; Frentiu & Chenoweth 2010). Third, precopulatory sexual selection, which may be a

key form of selection influencing the evolution of sex-biased gene expression (Ellegren & Parsch 2007; Harrison *et al.* 2015), has been directly measured along this latitudinal gradient (Rundle *et al.* 2008) and is known to vary in a nonclinal, population-specific manner. Finally, because *D. serrata* is endemic to eastern Australia, its underlying population genetic structure is less likely to represent multiple introductions and secondary contact events that can confound inferences of spatially varying selection in nonendemic species such as *D. melanogaster* and *Drosophila simulans* (Lack *et al.* 2015; Bergland *et al.* 2016).

In this study, we assessed the degree to which patterns seen in interspecific studies of *Drosophila* (i.e. elevated male-biased gene expression divergence and faster–X divergence of expression levels) are mirrored at the intraspecific level. Given the sampling scheme, we tested for sex differences in the modes of divergence (linear clinal vs. population specific) that might reflect differences in the forms of spatially varying selection. Using published data from other *Drosophila* studies, we also tested for parallel latitudinal divergence between species and continents that might illuminate common targets of selection.

Materials and methods

Biological samples, RNA extraction and microarray hybridization

The goal of our study was to estimate 'common garden' mean expression level for genes in each sex, rather than to estimate within population genetic variation. Flies were sampled from eight populations along the east coast of Australia, covering a straightline distance of approximately 2300 km, which spans much of the species natural range (Fig. 1). To preserve the natural genetic differences among populations and minimize adaptation to the laboratory, flies for each population were maintained as isofemale lines (David *et al.* 2005) ($n = 12$ for all populations with the exceptions of Airlie Beach, $n = 10$ and Cooktown, $n = 6$) until the gene expression assay. At this point, to ensure gene expression was measured on outbred flies, we crossed the isofemale lines within populations following a double-round-robin mating design that included reciprocal crosses by sex (Verhoeven *et al.* 2006; Stich 2009). For example, isofemale line 1 × isofemale line 2, isofemale line 1 × isofemale line 3, isofemale line 2 × isofemale line 3, isofemale line 2 × isofemale line 4, and so on. Owing to a smaller number of available lines, a triple round-robin mating design was used for Airlie and all possible pairwise crosses were performed for Cooktown. A total of 18 F1 crosses were randomly selected

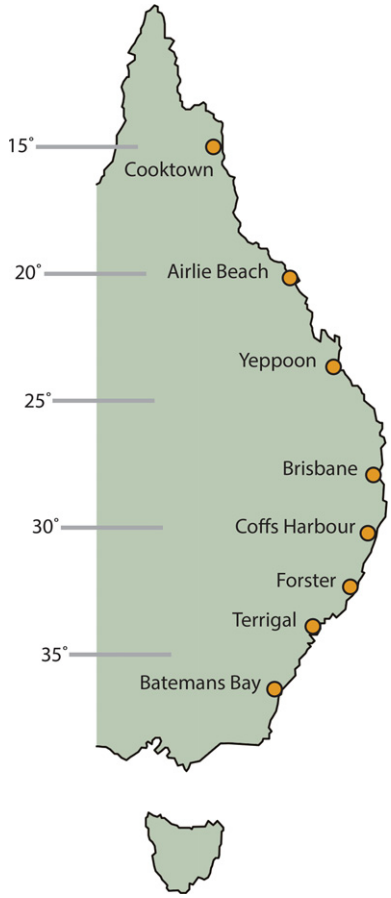

Fig. 1 Sampling locations of the eight natural populations of *Drosophila serrata* along the eastern Australian coastline. [Colour figure can be viewed at wileyonlinelibrary.com]

for RNA processing from each population with six crosses assigned to each of three biological replicates. Five flies were randomly selected from each cross to produce pools of 30 adult virgin flies (3 days old) per biological replicate. The samples were snap-frozen in liquid nitrogen without the use of CO_2 anaesthesia. Freezing began at 10:22 am and was completed by 1:40 pm. All flies were frozen in a random order with respect to sex and population. All flies were reared in 50-ml vials containing standard yeast medium at 25°C with a 12-hours day/night cycle, and adult flies were held in vials for 3 days in same sex groups of five before being frozen.

RNA extractions were performed using the TRIzol® (ThermoFisher) procedure followed by RNA isolation using RNeasy minikits®. cDNA synthesis, labelling, hybridization and microarray scanning were performed by the Centre for Genomics and Bioinformatics, Bloomington, Indiana. Quality control of the array data was performed via the BioConductor 'oligo package' using probe level models (Gentleman *et al.* 2004; Carvalho &

Irizarry 2010; Draghici 2012) and the experimental metrics report provided by NimbleGen. One presumed male sample from the Cooktown population was excluded due to a labelling error which reduced the data set from $n = 48$ to $n = 47$ hybridizations.

Custom microarray platform

A custom Nimblegen 12 × 135 K microarray was used to measure male and female gene expression of eight natural populations; the microarray design has been previously described (Allen *et al.* 2013). Briefly, a maximum of five probes per gene (mean = 4.99) were successfully designed for 11 631 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 (Frentiu *et al.* 2009) and Illumina RNA-seq-derived ESTs. Based on sequence comparisons to 12 other *Drosophila* species (McQuilton *et al.* 2012) and exclusion of orthologs using orthoDB (Waterhouse *et al.* 2013), it was assumed that each EST represented expression of a unique gene. The EST sequences used for microarray design purposes (length ≥ 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 *Drosophila serrata* (Frentiu *et al.* 2009). A total of 283 ESTs were shorter than the 200 bp minimum requirement of TSA and therefore could not be deposited; these are available directly from the authors. The chromosomal location of genes on this microarray has also previously been established (Allen *et al.* 2013).

Preprocessing

During quality control assessment, minor technical artefacts in the form of random spotting errors during microarray printing (Draghici 2012) were apparent on eight of the 47 microarrays. For this reason, each microarray was assigned a reliability weight using the *arrayw* procedure of the Bioconductor *limma* package (Ritchie *et al.* 2006). These weights were then used in the statistical models described in the next section. Raw gene expression measurements were $\log_2$-transformed to normality and then outlier probes within each sex were identified and omitted via Tukey's criteria (*t*-test *P*-value < 0.0005) on a probe-by-probe basis (Draghici 2012). The average expression of the two replicate probes was then calculated before mean summarization of each probe set. All subsequent analyses were performed on these mean summarized data.

Statistical analyses

Identification of sex-biased and sex-limited genes. In highly replicated experiments such as this, the use of statistical tests alone to classify sex bias can lead to genes with very small sex differences in expression being declared as sex-biased. Such small differences may not be biologically relevant (Stewart *et al.* 2010). For this reason, and to facilitate comparison with previously published studies (e.g. Ayroles *et al.* (2009); Hutter *et al.* (2008); Innocenti & Morrow (2010); Meiklejohn *et al.* (2003)), we classified genes as sex-biased if there was at least a twofold expression difference between the sexes, that is that expression was twice as high in one sex relative to the other, and the multiple-test-corrected *P*-value for a difference between the sexes was less than 0.05. Use of a lower 1.5-fold difference threshold (and multiple-test-corrected $P < 0.05$) resulted in very similar overall findings (Tables S3 and S4, Supporting information). We therefore only report on the twofold difference analyses in the main text. Sex bias was measured as mean $\log_2$-male − mean $\log_2$female expression. Mean male and mean female expression values were estimated across the entire data set using the UNIVARIATE procedure in SAS (Version 9.3, SAS Institute, Cary, NC) and fitting the array weights using the WEIGHT statement. Statistical differences between the sexes were assessed using the lm statement in R with array weights fitted using the weights argument (R Core Team 2016). We note that extraction of RNA from whole adult flies maximized the possibility of identifying sex-biased genes and that use of different tissues or developmental stages may result in different findings (Allen *et al.* 2013; Grath & Parsch 2016).

We assessed sex limitation in expression (also referred to as sex-specific genes) using a minimum expression threshold (Wang *et al.* 2006; Simon & Biot 2010; Draghici 2012). The threshold was based on the 20 000 random control probes present on each microarray (total 940 000) and set as the sex-specific mean expression level across all random probes plus two standard deviations, a value that allows maximum specificity (Bilban *et al.* 2002). Genes were classified as sex-limited if they exceeded their sex-specific threshold in one sex but not in the other. Only genes that were expressed in both sexes were considered as potentially sex-biased.

Divergence in gene expression. Our first goal in assessing divergence in expression was to determine how many genes have diverged in a linear latitudinal pattern as opposed to a significant but nonlinear, population-specific pattern. To achieve this, we fitted the linear model;

$$\text{expression} = \text{latitude} + \text{population} + \text{error}, \quad (1)$$

where latitude (measured as degrees south to four decimal places) was fitted as a continuous factor and population a categorical factor. We fitted the terms sequentially; latitude followed by population using sequential sums of squares. This model provides us with the opportunity to test for clinal variation plus any divergence among populations that departs from linearity while simultaneously accounting for any aforementioned clinal effect. The model was fit using the GLM procedure of SAS version 9.3 (SAS Institute 2013). Array weights were also incorporated into the model via the WEIGHT statement. Multiple-test corrections were conducted using a false discovery rate of 5% to each model term (Benjamini & Hochberg 1995) via the R/p.adjust() function (method='BH'). Model 1 needed to be fit using sequential sums of squares, and so it was not possible to fit a mixed effects model that incorporated gene-specific effects of chip. For this reason, we analysed gene expression as residuals from a gene-specific random effects model that statistically removed the random effect of chip (expression = chip + error). The random effect model was fit via the MIXED procedure in SAS. The entire analysis was performed on males (male-expressed genes: unbiased, male- and female-biased and male-limited) and females (female-expressed genes: unbiased, male- and female-biased and female-limited) separately.

To compare effect sizes between sexes and different types of sex-biased genes, we compared the transcriptome-wide distributions of R^2 values from model 1 using Mann–Whitney U-tests. We conducted these tests on all the effect size distributions for genes regardless of statistical significance to avoid ascertainment bias inherent when applying threshold-based significance testing. We used hypergeometric tests [R/phyper()] to assess nonrandom patterns in the numbers of genes diverging in different modes (clinal vs. population specific) according to sex bias, sex of expression and chromosomal location.

Gene ontology (GO) term enrichment analysis. To determine whether genes underlying specific biological functions were more likely to have diverged than others, we performed gene ontology (GO) term enrichment analysis after assigning functions to *D. serrata* ESTs based on homolog identification as follows (Table S1, Supporting information). Each EST was linked to an annotated feature from the draft *D. serrata* genome assembly (Allen *et al.* 2017; doi: 10.1101/090969) via tblastx (NCBI standalone blast version 2.3.0+). All ESTs were successfully linked to a *D. serrata* feature with a median e-value of $3.76e^{-137}$. Then, each *D. serrata* feature sequence was classified as a putative *Drosophila melanogaster* homolog using the method of reciprocal best hits (Tatusov *et al.* 1997; Bork *et al.* 1998; Moreno-Hagelsieb & Latimer 2008) with *D. melanogaster* coding sequences (tBLASTx default settings) obtained from FlyBase (genome version 6.05) (Drosophila 12 Genomes Consortium 2007; McQuilton *et al.* 2012). The *D. melanogaster* gene GO terms were then assigned to the *D. serrata* ESTs and used for enrichment analysis. To allow for divergent genes to be identified, tBLASTx with a liberal e-value threshold of 10 was applied; however, in practice the median e-value was $1.20e^{-162}$. This method successfully identified 10 555 ESTs on the microarray (91%) as *D. melanogaster* homologs. Annotation of the *D. serrata* genome is currently incomplete with many genes remaining to be annotated. In some cases, annotated features are in reality multiple genes that will await correction via manual curation (Yandell & Ence 2012). As a consequence, we refer to our *D. serrata* annotations as homologous to *D. melanogaster* as opposed to being strict one-to-one orthologs. Gene ontology enrichment analysis was performed using g:Profiler (Reimand *et al.* 2016) with ordered gene lists by *P*-value and a false discovery rate of 5% (Benjamini & Hochberg 1995). The same approach was used to identify *D. simulans* homologs using genome version 2.02 obtained from FlyBase, and 10 493 ESTs on the microarray (90%) were identified as *D. simulans* homologs.

Correlated patterns of divergence in males and females. We assessed whether gene expression divergence was correlated between males and females. For all co-expressed genes (those expressed in both sexes), we estimated the Pearson's product–moment correlation between the population mean vectors for males and females, $r_{pop(m,f)}$. High values of $r_{pop(m,f)}$ indicate that males and females have diverged in similar ways along the gradient, whereas low correlations suggest divergence is sex specific. We compared the distribution of $r_{pop(m,f)}$ values between genes that showed either divergence in both sexes, males only or females only. We also examined the distributions of genes showing linear clinal as opposed to population-specific divergence.

To better assess changes in sexual dimorphism among populations, we analysed all co-expressed genes using supplementary combined sex analyses. To test for sex-specific population divergence, we used the following ANOVA model:

$$\text{expression} = \text{sex} + \text{population} + \text{sex} \times \text{population} + \text{error}, \quad (2)$$

The significance of the sex × population interaction was used to test for sex-dependent divergence and multiple-test-corrected to a false discovery rate of 5% (Benjamini & Hochberg 1995). Owing to insufficient degrees of freedom, it was not possible to fit population and

latitude simultaneously in model 2 as we did for the single sex analyses in model 1. Therefore, we fit a separate version of model 2 where the categorical population term was replaced with the continuous factor of latitude.

Results

Sex-biased genes

Using a custom expression array platform, we analysed expression at 11 631 *Drosophila serrata* genes. All but 295 of the genes analysed passed the minimum expression threshold in at least one sex. These were therefore excluded from further analysis. Moreover, although most genes were expressed in both sexes (9934, 85%), there were far more male-limited (1357, 11.7%) than female-limited genes (45, 0.4%). Of the genes that were expressed in both sexes (co-expressed genes), use of a twofold $\log_2$(expression) threshold to detect sex bias revealed that there were slightly more female- (2648, 22.8%) than male-biased (2456, 21.1%) genes. A similar result was found when using a 1.5-fold threshold to define sex bias (Table S3, Supporting information). Overall, our results are consistent with studies of other *Drosophila* species, where the numbers of sex-biased genes are typically reported as sex-biased plus what we have classified here as sex-limited (Zhang *et al.* 2007).

Expression divergence is stronger in males than in females

In our analysis of male and female transcriptome divergence among the eight populations, we tested each gene simultaneously for both (i) predictable linear associations with latitude, hereafter coined 'clinal divergence', and (ii) residual population-specific divergence from the latitudinal trend (see methods). In broad terms, we saw a greater fraction of the male transcriptome divergence among populations than the female transcriptome, limited overlap between sexes in those genes that diverged, and a tendency towards population-specific divergence along the latitudinal gradient in males (Fig. 2). For males, a total of 1483 genes (13.1%) were significant for either the linear effect of latitude, the categorical effect of population, or both (Table 1. FDR < 5%). For these genes, there was a relatively uneven distribution in the pattern of divergence: over half (781, 53%, Table 1) showed only a population-specific pattern of divergence with a significant main effect of population but not latitude, whereas only a third (482, 33%, Table 1) showed a linear clinal association with latitude without a significant population effect. A total of 220 genes showed both a latitudinal and population effect (14% Table 1), suggesting overall clinal variation but with some residual population-specific divergence.

Far fewer genes diverged significantly when expressed in females, with only 805 (8.1% of genes expressed in females), showing divergence at FDR <5%. Among these, similar numbers showed linear clinal (Table 1: 325, 40%,) and population-specific (Table 1: 337, 42%) divergence, although the overlap between the modes of divergence was similar to that seen in males (143, 18%). In a pattern suggestive of extensive sex-specific divergence, there was limited overlap in the identity of genes that diverged significantly in males and females: only 182 genes diverged significantly in both males and females (9.4% of diverged genes that were expressed in both sexes).

We also compared the distribution of linear model effect sizes between males and females using the R^2 values. For all but sex-limited genes, which are by definition a nonoverlapping set of genes between sexes, the proportion of variance explained by latitude and population combined (model 1) was far greater when a gene was expressed in males compared with when it was expressed in females (Fig. 3). Moreover, the elevation in effect sizes appeared strongest for male-biased genes.

As with other *Drosophila* studies (Catalan *et al.* 2012; Zhao *et al.* 2015), there was bias in the direction of latitudinal clines. For genes showing a significant effect of latitude in males, expression tended to increase at higher latitudes (southwards) more often than it decreased. This skew was significant using binomial tests on the sign of regression coefficients (males: 462 positive vs. 239 negative; binomial $P < 2.2 \times 10^{-16}$). Directional bias was far more pronounced in males than it was in females, where the effect was marginally nonsignificant (females: 251 positive vs. 210 negative; binomial $P = 0.062$). There is a possibility that this result is related to a body size cline that has been reported in *D. serrata* (Hallas *et al.* 2002) where body size increases as latitude increases. However for this to occur, tissue composition of the flies would also have to scale nonisometrically with body size (Montgomery & Mank 2016), a question that is yet to be answered.

Divergence is enriched for male-biased genes

Previous macroevolutionary studies have reported greater expression divergence in male- than female-biased and unbiased genes (Ellegren & Parsch 2007), as have some intraspecific comparisons of *D. melanogaster* populations (Meiklejohn *et al.* 2003; Hutter *et al.* 2008; Zhao *et al.* 2015) but see Muller *et al.* (2011). In general agreement, divergence in *D. serrata* was significantly enriched for male-biased genes. Proportionally, far more male-biased genes diverged among the sampled

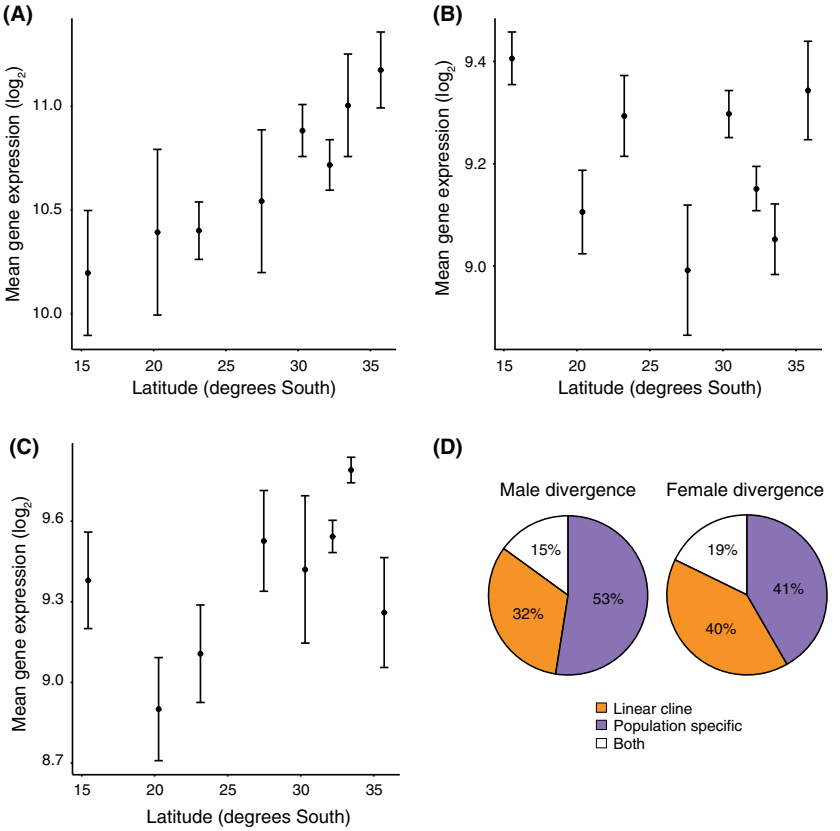

Fig. 2 Example plots displaying different types of divergence in gene expression. All examples are from males and the error bars are 95% confidence intervals. Latitude increases from left (Cooktown) to right (Batemans Bay). (A) EST3327 diverged in a linear clinal pattern. (B) EST37600 diverged with a population-specific pattern. (C) EST25624 had a significant main effect of latitude and population. (D) Pie charts for male and female divergence displaying the proportion of genes that diverged for each mode of divergence. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1 Number of genes with significant male or female expression divergence among eight populations of *Drosophila serrata* sampled along a latitudinal gradient. Gene counts are arranged by sex of expression and sex bias class. Divergence mode corresponds to significance being detected in model 1 for either the latitude and/or population effects (FDR <0.05). Percentages are given in parentheses and correspond to fraction of significant genes within each sex bias class relative to the number analysed. Significance values indicate significant enrichment is indicated against other classes of sex bias using hypergeometric tests

		Divergence mode			
Sex bias	*n* Genes	Clinal	Population specific	Both	Total (either)
Males					
Unbiased	4830	243 (5.0)**	287 (5.9)	88 (1.8)	618 (12.8)
Female-biased	2648	57 (2.2)	101 (3.8)	26 (1.0)	184 (6.9)
Male-biased	2456	148 (6.0)**	304 (12.4)**	75 (3.1)**	527 (21.5)**
Male-limited	1357	34 (2.5)	89 (6.3)	31 (2.3)	151 (11.1)
All genes	11 291	482 (4.3)	781 (6.9)	220 (1.9)	1483 (13.1)
Females					
Unbiased	4830	152 (3.1)	120 (2.5)	40 (0.8)	312 (6.5)
Female-biased	2648	78 (2.9)	62 (2.3)	20 (0.8)	160 (6.0)
Male-biased	2456	89 (3.6)	151 (6.1)**	82 (3.3)**	322 (13.1)**
Female-limited	45	6 (13.3)**	4 (8.9)*	1 (2.2)	11 (24.4)**
All genes	9979	325 (3.3)	337 (3.4)	143 (1.4)	805 (8.1)

*$P < 0.05$; **$P < 0.005$.

populations than other types of genes. There was a significant enrichment of male-biased genes for divergence in both males (Table 1: 527, 21.5%, hypergeometric test: $P < 1 \times 10^{-8}$) as well as females (Table 1: 322, 13.1%, hypergeometric test: $P < 1 \times 10^{-8}$). A similar result was observed for the 182 genes that diverged in both sexes

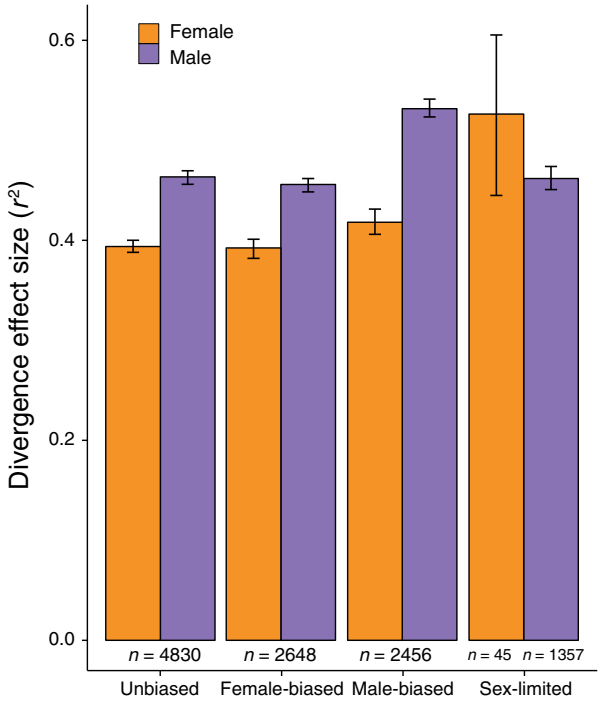

Fig. 3 Transcriptome-wide effect size estimates (median model 1 R^2 values) for the combined effects of latitude and population for all genes analysed according to sex bias category. Error bars are 95% confidence intervals of the median based on 10 000 pseudosamples of the original data. Numbers of genes analysed in each sex and class also appear below the bars. [Colour figure can be viewed at wileyonlinelibrary.com]

where an excess of male-biased genes was found (35%, hypergeometric test: $P = 5.50e^{-04}$). We also observed significant enrichment of female-limited (Table 1: 11, 24.4%, hypergeometric test: $P < 1.7 \times 10^{-4}$) but not male-limited genes (Table 1: 151, 11.1%, hypergeometric test: $P = 0.98$). Enrichment for male-biased genes was present across all divergence modes: linear clinal, population specific and both (Table 1), but was highest in the analysis of males for genes displaying a purely population-specific pattern (Table 1). Female-biased genes were consistently the most underrepresented class among diverging genes regardless of sex. These results were qualitatively identical when a 1.5-fold threshold was used to classify sex bias (Table S3, Supporting information).

Divergence is correlated between males and females

We saw limited overlap in the genes showing significant divergence in males and females. Such a pattern suggests there could be extensive changes in either the degree or direction of sex bias across these natural populations. To examine changes in sex bias, we performed supplementary analyses of all sexually co-expressed genes ($n = 9934$), fitting a gene-specific linear model that included the main effects of population, sex and their interaction as fixed effects. Here, a significant sex × population interaction would signal a change in either degree or direction of sexual dimorphism among the sampled populations. The number of genes with a significant sex × population interaction was modest (366 at FDR < 5%). We also tested a model including sex and latitude (i.e. expression = sex + latitude + sex × latitude + error) and again saw a small number of interactions (sex × latitude: 45 at FDR < 5%).

A low number of significant interactions appear somewhat at odds with the separate sex analyses where many genes were found to have diverged in one sex only. While it may be the case that the combined sex models lacked statistical power to detect sex-specific divergence via interaction effects, it was also possible that positive genetic correlations between males and females for gene expression (e.g. *D. melanogaster* mean $r_{mf} = 0.4$; Griffin *et al.* 2013) may inhibit sex-specific divergence despite widespread sex-specific selection. Moreover, if divergence were also consistently stronger in one sex than another, as we saw for males in the separate sex analysis (Fig. 2), there would be limited overlap in genes reaching significance in the separate sex analyses. To test this idea, we calculated the correlation between male and female population means across the eight populations, $r_{pop(m,f)}$. Divergence was indeed most commonly positively correlated between the sexes (Fig. 4). Genes showing significant divergence typically had much higher $r_{pop(m,f)}$ values and it was maximal for the 182 genes that diverged significantly in both sexes. This overall pattern suggests that while divergence in co-expressed genes is usually correlated between sexes, it tends to occur to a greater degree in males.

Sex differences in X-Autosome bias

Although there is considerable evidence for a faster-X effect from macroevolutionary comparisons of *Drosophila* species (Ellegren & Parsch 2007; Parsch & Ellegren 2013), whether the same is true over microevolutionary timescales is unclear, as both faster-X (Meisel *et al.* 2012) and slower-X effects (Hutter *et al.* 2008) have been reported in *D. melanogaster*, a recent study failed to find either a faster- or slower-X effect (Zhao *et al.* 2015). In *D. serrata*, there were sex differences in the representation of X-linked genes among the sets of significantly diverged genes; however in males, we did not find any evidence that X-linked genes diverged among the eight natural populations of *D. serrata* more often than autosomal genes. Instead, X-linked genes were significantly

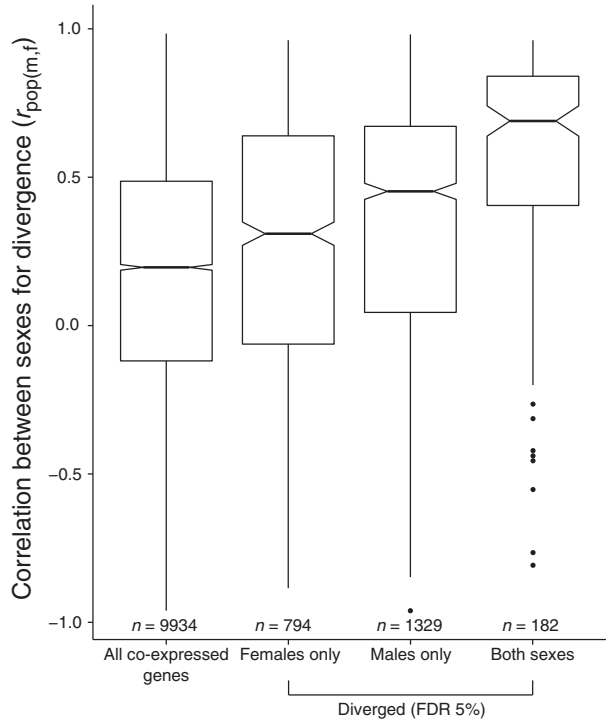

Fig. 4 Distribution of the among population correlation between male and female mean gene expression, $r_{pop(m,f)}$, for genes showing any form of significant population divergence (model 1) in males, females and both sexes. Also shown are the genomewide estimates for all co-expressed genes regardless of statistical significance.

underrepresented (Table 2). In males, only 7.3% of X-linked genes analysed diverged despite them representing over 14% of the transcriptome, a significant deficit according to a hypergeometric test (test of deficit $P = 3.1 \times 10^{-16}$). The paucity of X-linked gene divergence was present regardless of the pattern of divergence (clinal: hypergeometric test deficit $P = 1.5 \times 10^{-7}$; population specific: $P = 4.8 \times 10^{-6}$; and both: $P = 2.2 \times 10^{-5}$). Because elevated X-linked divergence of gene expression between some *Drosophila* species is strongest for male-biased genes (Meisel *et al.* 2012), we considered whether this may also be the case in *D. serrata*, despite the paucity of X-linked divergence in males overall. However, when we tested for an enrichment of X-linked genes across the different sex bias classes and classification thresholds (twofold vs 1.5-fold), the deficits remained for all sex bias classes except male-limited where the deficit was marginally nonsignificant (hypergeometric test: $P = 5.48e^{-02}$) (Table S4, Supporting information).

In contrast to the lack of X-linked divergence in males, for females there was significant enrichment of X-linked genes, comprising 19% of significantly divergent genes compared with 16% in the analysed

Table 2 Chromosomal distribution of genes that diverged significantly among populations (FDR <0.05) for males and females. Significance values correspond to hypergeometric tests for significant enrichment (*) or deficits (†) of genes. Genes in the unknown or unplaced (U) categories were not tested and were not included in the total sample sizes when performing hypergeometric tests

		Divergence mode			
Chromosome	*n* Genes	Clinal	Population specific	Both	Total (either)
Males					
X	1657	35††	73††	13††	121††
2L	1996	118**	135	39	292*
2R	2217	104	154	50	308
3L	2118	92	152	49	293
3R	2697	111	217**	61	389*
4	72	4	5	1	10
Y	6	0	0	0	0
U	38	1	5	0	6
Unknown	490	17	40	7	64
All genes	11 291	482	781	220	1483
Females					
X	1558	64*	51	31*	146*
2L	1729	62	52	30	144
2R	1973	64	62	25	151
3L	1889	54	60	20	134
3R	2388	69	98*	26	193
4	71	1	0	0	1†
U	31	1	1	2	4
Unknown	340	10	13	9	32
All genes	9979	325	337	143	805

*Enrichment $P < 0.05$; **enrichment $P < 0.005$.
†Deficit $P < 0.05$; ††Deficit $P < 0.005$.

transcriptome (Table 2; hypergeometric test of enrichment, $P = 1.4 \times 10^{-2}$). Interestingly, the enrichment of X-linked genes was absent from the population-specific divergence set (Table 2; hypergeometric test, $P = 1.3 \times 10^{-1}$) and was only seen for genes showing either linear latitudinal (Table 2; hypergeometric test, $P = 1.6 \times 10^{-3}$) or both types of divergence (Table 2; hypergeometric test, $P = 1.6 \times 10^{-2}$). Interestingly, when broken down by sex bias class, we saw that X chromosome enrichment in females was only significant for female-biased genes (Table S4, Supporting information; hypergeometric test, $P = 9.8 \times 10^{-3}$). A similar observation was made when a 1.5-fold sex bias threshold was used in place of the twofold threshold (Table S4, Supporting information).

Divergence was also nonrandomly distributed across the four major autosomal arms in *D. serrata* (Table 2). In males, there was significant enrichment of genes on 2L and 3R but in different divergence modes. Genes on 2L were enriched in the linear divergence set (Table 2:

hypergeometric test, $P = 6.8 \times 10^{-5}$), whereas genes on 3R were overrepresented in the population-only set (hypergeometric test, $P = 2.05 \times 10^{-3}$). Similarly, in females we observed enrichment for 3R in the population-only divergence set (hypergeometric test, $P = 9.5 \times 10^{-3}$) but the enrichment of genes on 2L for linear divergence in females was not significant (hypergeometric test, $P = 1.8 \times 10^{-1}$). Similar nonrandom patterns have been seen in *D. melanogaster*, which may be due to segregating chromosomal inversions (Zhao *et al.* 2015). However, owing to a lack of genomic information for *D. serrata*, we were not able to assign genes to inversions.

Gene ontology analysis of divergent genes

Gene ontology enrichment of the divergent genes using g:Profile (Reimand *et al.* 2016), revealed sex differences in divergent gene function and also functional differences between the different modes of geographical divergence. While full results are available in Table S4, Supporting information, some highlights are outlined below. For clinal divergence in males, we saw enrichment of the term *response to ethanol* (p.adj = $3.63e^{-02}$), noteworthy given known divergence in the alcohol dehydrogenase gene (Adh) in *D. melanogaster* (Oakeshott *et al.* 1982; David *et al.* 1989; Berry & Kreitman 1993). Terms related to metabolism were also enriched including *digestion* (p.adj = $1.00e^{-02}$), *carbohydrate metabolic process* (p.adj = $4.43e^{-05}$), *lipid catabolic process* (p.adj = $2.13e^{-02}$) and *proteolysis* (p.adj = $5.82e^{-02}$), similar to a previous report in *D. melanogaster* males (Hutter *et al.* 2008). Male population-specific divergence was enriched for *cuticle development* (p.adj = $1.58e^{-03}$). Insect cuticles perform many important functions such as providing structure and muscle attachment for locomotion, protecting against xenobiotics and infection, and assisting in desiccation resistance (Moussian 2010). Genes showing both clinal and population-specific divergence were enriched for a single term, the molecular function *immune response* ($P = 1.78e^{-04}$, p.adj = $3.45e^{-05}$).

In females, clinal divergence was enriched for terms related to oogenesis, in particular *egg coat formation* (p.adj = $6.31e^{-04}$). Clines in traits related to oogenesis such as ovariole number and egg size have been documented in a wide range of species (Adrion *et al.* 2015) including *D. melanogaster* (Azevedo *et al.* 1996). Female population-specific divergence was enriched for *catalytic activity* (p.adj = $5.00e^{-02}$).

Parallel divergence with other Drosophila species

Between-species overlap in the genes diverging across latitudinal gradients may strengthen evidence for climatic adaptation. We took advantage of a recent study of divergence in male gene expression between a tropical and temperate population of *D. melanogaster* and *D. simulans* (Zhao *et al.* 2015) and compared gene lists for divergence with male *D. serrata*. A total of 11 291 of the *D. serrata* ESTs that were expressed in males were linked to 8294 unique *D. melanogaster* genes. Of these 8294, 160 diverged in both species which represented, 12.5% of the 1283 that diverged in *D. serrata* and 25.5% of the 783 that diverged in *D. melanogaster*, and this degree of overlap was greater than expected by chance (hypergeometric test, $P = 5.7e^{-05}$). GO term enrichment for these overlapping genes revealed overrepresentation of numerous biological processes (Table S5, Supporting information), including *regulation of circadian rhythm* (p.adj = $4.57e^{-02}$), *mating behaviour* (p.adj = $1.34e^{-02}$), *response to ethanol* (p.adj = $6.97e^{-03}$) and several metabolic process terms such as *digestion* (p.adj = $4.68e^{-02}$), *lipid metabolic process* (p.adj = $4.94e^{-02}$) and *cellular amino acid catabolic process* (p.adj = $4.68e^{-02}$). In addition, several noteworthy enriched molecular functions were *oxidoreductase activity* (p.adj = $4.26e^{-02}$) and *structural constituent of cuticle* (p.adj = $4.12e^{-02}$).

For the comparison between *D. serrata* and *D. simulans*, 11 291 of the male-expressed ESTs assessed in *D. serrata* were linked to 8246 unique *D. simulans* FlyBase gene ids. Of these, 174 diverged in both species, which equates to 13.7% of the 1271 that diverged in *D. serrata* and 19.6% of the 886 that diverged in *D. simulans*, a proportion that was greater than expected by chance (hypergeometric test, $P = 2.0e^{-04}$). GO term enrichment of the common genes that diverged in both *D. serrata* and *D. simulans* included a single term, *structural constituent of cuticle* (p.adj = $5.00e^{-02}$).

Discussion

We have compared male and female gene expression divergence along a latitudinal gradient covering a large fraction of the endemic distribution of *Drosophila serrata*. Our analyses revealed marked sex differences in the frequency, mode and strength of geographical divergence. As well as sex differences, strong differences were also seen between sex bias classes, with far more male-biased genes diverging than female-biased genes regardless of whether they were expressed in males or females. In males, divergence was not enriched for X-linked genes, and instead, a significant deficit was observed. In contrast, for genes expressed in females, divergence was enriched for X-linked genes with the effect strongest for female-biased genes. Finally, we found evidence for gene overlap with *D. simulans* and *D. melanogaster* spanning the east coast of America, indicating a degree of parallel adaptation at the level of gene expression in these species. These results provide

insight into the evolution of sex bias in gene expression in response to both macroecological (clinal) and microecological (population specific) variation. We discuss these key findings in further detail below.

Strong sex differences in clinal and nonclinal divergence

While some genes showed both linear latitudinal and population-specific divergence modes, the numbers showing clinal divergence in each sex were similar in proportional terms (14% in males and 17% in females). However, males and females differed in the relative number of genes showing only one mode of divergence (clinal or population specific). While approximately equal numbers were detected for both modes in females, far more genes (1.6 times) diverged in a population-specific, rather than linear clinal pattern in males. Because many abiotic factors tend to covary predictably with latitude (Endler 1977), genes for which divergence scaled systematically with latitude are consistent with the operation of clinally varying natural selection. For example, several genes associated with cold acclimation diverged in a clinal pattern in males as did several genes associated with circadian rhythm in both sexes (Table S5, Supporting information), including a homolog of the genes *homer*, an essential protein for the regulation of circadian sleep/wake cycle (Naidoo *et al.* 2012), and *takeout*, a gene implicated in the circadian control of feeding behaviour (So *et al.* 2000).

Population-specific divergence patterns on the other hand suggest less predictable forms of selection. Given the abundance of population-specific effects in males, an obvious candidate form of selection is sexual selection. Because sexual selection fundamentally involves biotic interactions, it may be less influenced by abiotic ecological factors (Andersson 1994) and may therefore be more likely to vary in a population-specific manner (Gosden & Svensson 2008). For example, it has previously been shown that sexual selection on *D. serrata* cuticular hydrocarbons (CHCs) varies spatially along this latitudinal gradient but does not always covary systematically with latitude; that is for some traits, sexual selection is population specific (Rundle *et al.* 2008). Consistent with this, we observed population-specific enrichment in males for the GO term *cuticle development* (Table S5, Supporting information). In further support of the possibility that population-specific divergence reflects sexual selection was the finding that, while enrichment for the biological process *sex comb development* was marginally nonsignificant after correction for multiple tests, the gene *sex combs extra* (Table S5, Supporting information), a polycomb group gene required for proper development of adult sex combs (Simon *et al.* 1992), did diverge in a population-specific manner.

Although genetic drift has been excluded as a major factor shaping clinal differentiation for some traits in *D. serrata* (Chenoweth & Blows 2008), it cannot yet be excluded for our analyses of divergence in gene expression. Previous *D. serrata* population genetic surveys across the sampled range showed quite weak levels of genetic differentiation. One showed significant, but weak, isolation by distance (Chenoweth & Blows 2008), which would predict some clinal divergence in expression by chance alone, whereas no such pattern was detected in an earlier study (Magiafoglou *et al.* 2002). An interesting argument against genetic drift in this study is provided by the divergence patterns of sex-limited genes. For example, because male-limited genes are not under selection in females (Gershoni & Pietrokovski 2014), male-limited genes are more exposed to genetic drift than co-expressed male-biased genes and likely even more so than unbiased genes. The finding that sex-limited genes did not diverge more often than co-expressed genes to some extent weakens the case for a major role of drift, as does the observation of parallel divergence with other species. Notwithstanding, more detailed population genomic studies will be required to determine the underlying population structure of the cline.

Male-biased divergence

Sex-biased gene expression is ubiquitous in dioecious species and its evolution has received significant empirical attention. Of particular interest is the finding that sex-biased genes, especially male-biased genes, appear to diverge at an increased rate in a wide range of species (Ellegren & Parsch 2007; Parsch & Ellegren 2013), a result clearly replicated in both sexes of *D. serrata* (Table 1). In males, approximately 2.9 times more male-biased genes diverged than female-biased genes, and in females approximately twice as many male-biased genes diverged than female-biased genes. An interesting explanation for the excessive divergence in male-biased genes is that, in general, selection might be stronger on male expression traits than female expression traits. For instance, male-biased genes most likely affect male more than female fitness (Connallon & Clark 2011) and evolutionary theory has long predicted that selection may be stronger on males than females due largely to sexual selection on males (Manning 1984; Kodric-Brown & Brown 1987; Whitlock & Agrawal 2009; Agrawal 2011), an idea supported by mutation accumulation experiments in *Drosophila* (Mallet *et al.* 2011; Sharp & Agrawal 2013). Our finding that

geographical divergence is enriched for male-biased genes, which are likely more important for male fitness than female fitness, provides further support for the hypothesis that males are perhaps under stronger selection than females.

Additional support for the idea of stronger selection on males comes from our analysis comparing the strength of expression divergence between males and females. We found that geographical divergence in males was indeed greater than females on a transcriptome-wide scale (Fig. 2). This sex difference was greatest for male-biased genes and was of a similar magnitude for both female-biased and unbiased genes. If divergence strength is associated with the strength of selection, this finding also suggests that spatially divergent selection among *D. serrata* populations may be stronger on males than on females.

Limited overlap between males and females in the genes showing divergence suggests that there may be substantial spatial variation in sex-specific selection across the sampled populations. However, we found a general paucity of significant interactions between sex and population (or latitude) when analysing the sexes together. It is possible that, despite variation in sex-specific selection, population divergence in sex bias has been constrained by positive genetic correlations between males and females, r_{mf} (Lande 1980, 1987). In *D. melanogatser*, gene expression is largely positively correlated between the sexes (Griffin *et al.* 2013). If this is also the case in *D. serrata*, then sex-specific divergence may be constrained and difficult to detect statistically regardless of the strength of sex-specific selection. For example, if selection for divergence was much stronger on males than females, but r_{mf} was also high, divergence would be of a similar direction and magnitude in males and females due to correlated responses despite the difference in selection strength. Although we were not able to measure r_{mf} in this experiment, we measured the intersexual divergence correlation, $r_{pop(m,f)}$, and found that it was most often positive; more so for genes that diverged in both sexes followed by male-biased genes and then female-biased genes (Fig. 4). Such correlated divergence, despite many genes apparently diverging in males only (in terms of statistical significance), indeed suggests that sex-specific adaptation in gene expression could be constrained by pleiotropy between sexes (Griffin *et al.* 2013; Innocenti & Chenoweth 2013). However, there is some evidence that cross-sex genetic covariances tend to vary across populations (Barker *et al.* 2010; Gosden & Chenoweth 2014), and therefore how such constraints manifest would be an interesting starting point for future studies.

X/Autosome bias

Comparisons between *Drosophila* species have revealed that X-linked genes often diverged to a greater extent than autosomal genes in terms of coding sequence (Charlesworth *et al.* 1987; Vicoso & Charlesworth 2006) and in some cases, expression levels (Llopart 2012; Meisel *et al.* 2012), coined the 'faster-X' effect (Betancourt *et al.* 2002). However, evidence of faster-X effects for gene expression patterns is inconsistent in comparisons between populations of a single species (Hutter *et al.* 2008; Zhao *et al.* 2015). In males, we found no numerical enrichment of X-linked genes and in fact the opposite was the case: X-linked genes were significantly underrepresented. A similar result was seen for male gene expression in a comparison between two *D. simulans* populations (Zhao *et al.* 2015) and between two *D. melanogaster* populations (Hutter *et al.* 2008). However, the *D. melanogaster* result was not replicated in a second study of other populations (Zhao *et al.* 2015). In contrast to the absence of faster-X effects in males, expression divergence in *D. serrata* females was enriched for X-linked genes.

One intriguing hypothesis to explain the joint observations of reduced X-linked divergence in males and elevated X-linked divergence in females is the hyperexpression of X-linked genes in female *D. serrata*. Female *D. serrata* show a pattern of general hyperexpression of the X chromosome that exceeds autosomal expression (Allen *et al.* 2013). This could expose X-linked genes to stronger selection when expressed in females (Pal *et al.* 2001). Thus, while the observed patterns are consistent with stronger overall selection on males, it may be the case that X chromosome hyperexpression leads to stronger selection at X-linked loci in females, thereby creating a concomitant deficit of X-linked divergence in males relative to the stronger divergence of autosomal genes. Support for this explanation comes from the observation that X chromosome enrichment was strongest for female-biased genes but weaker and marginally nonsignificant for male-biased and borderline significant for unbiased genes (Table S1, Supporting information). Hyperexpression of X-linked genes, although less pronounced, has been reported for other *Drosophila* species (Gupta *et al.* 2006; Sturgill *et al.* 2007; Zhang *et al.* 2007) and the red flour beetle (*Tribolium castaneum*) (Prince *et al.* 2010), although its relationship to X chromosome evolution is as yet unknown. More studies will be needed to determine whether 1) intraspecific faster- or slower-X effects on gene expression are common and 2) whether sequence evolution of X-linked genes in species with X hyperexpression differs to those without it.

Parallel divergence between Drosophila species

Common genes that have diverged among populations in different species along comparable latitudinal gradients provide a strong indication that these genes are under spatially varying selection (Futuyma 2005; Zhao *et al.* 2015). Comparing our results with a previous study of latitudinal gene expression divergence both *D. melanogaster* and *D. simulans* (Zhao *et al.* 2015), we found significant overlap in the genes that diverged and GO term analysis implicating multiple biological processes likely under spatially divergent selection. These include genes associated with circadian rhythms in comparisons with *D. melanogaster*. This is an expected result, given that circadian rhythms are likely under strong natural selection due to their ability to tailor behaviours and physiological responses to environmental changes that are dependent on the time of day (Panda *et al.* 2002).

We also found enrichment for genes related to the cuticle in both species comparisons. The insect cuticle performs many important functions such as protection, structure for locomotion and desiccation resistance (Gibbs 1998, 2002; Moussian 2010). However, clines in desiccation resistance have been reported for some *Drosophila* species (Hoffmann & Harshman 1999), which suggest a selected function of the cuticle genes. Unique to the *D. melanogaster* comparison, we found enrichment for genes associated with lipid and protein metabolism. This is perhaps reflective of the finding that metabolism increased clinally with latitude on the east coast of Australia in *D. melanogaster*, likely due to changes in average temperature (Berrigan & Partridge 1997). Lastly, we found enrichment for genes related to mating behaviour and reproduction in the *D. melanogaster* comparison, traits that are likely under sex-specific selection (Andersson 1994; Futuyma 2005). Overall, while the evidence for parallel adaptation in gene expression between continents and species strongly points to shared selective regimes and abilities to respond to selection between the species, there is also a great deal of species specificity in the responses.

Conclusion

Our study has exposed marked sex differences in the microevolutionary divergence of gene expression across macro- and micro-ecological scales. The patterns observed suggest a history of stronger divergence on males than females. As many of the genes that diverged in a population-specific manner were male-biased, and tended to diverge predominantly in males, it suggests that divergence could be driven by male sexual selection that varies over microecological scales. While we have measured transcript abundance here, it will be interesting to see whether, as is the case with interspecific divergence patterns, similar patterns are seen in coding sequence variation along this latitudinal gradient. Several studies have reported considerable changes in sex bias between species of *Drosophila* (Zhang *et al.* 2007) with up to 20% of sex-biased genes showing a gain, loss or reversal in sex bias between *Drosophila melanogaster* and *Drosophila simulans* (Ranz *et al.* 2003). Despite our finding that gene expression diverged more often and to a greater degree in male *Drosophila serrata* (Fig. 2), we found little evidence for changes in the degree of sex bias along this cline. This contrast between macro- and micro-evolutionary patterns may be caused by genetic constraints to the evolution of sex-biased gene expression (Mank *et al.* 2008; Griffin *et al.* 2013; Innocenti & Chenoweth 2013) that require macro-evolutionary timescales to overcome.

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Data accessibility

All gene expression data have been deposited in the Gene Expression Omnibus (GSE90733), which include both raw and preprocessed data in an as analysed state.

Conceived and designed the experiments: S.A., R.B., C.S. and S.C. Provided biological samples: C.S. and S.C. Performed the experiments: S.A. Analyzed the data: S.A and S.C. Wrote the paper: S.A and S.C. Edited the paper: S.A., R.B., C.S. and S.C.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Classification of *Drosophila serrata* ESTs as either *Drosophila melanogaster* or *Drosophila simulans* homologs.

Table S2 Full lists of ESTs that diverged for each sex and divergence mode plus sex-dependent divergence.

Table S3 Number of genes with significant male or female expression divergence broken down by sex-bias type and divergence mode when using either a 2-fold or 1.5-fold expression difference to define sex-bias.

Table S4 Numbers of genes showing significant divergence (FDR<0.05) among populations in *Drosophila serrata* broken down by sex-bias class, divergence mode and chromosome.

Table S5 G:Profiler Gene Ontology term analysis of divergent genes (FDR<0.05) broken down by sex and divergence mode.

Table S6 G:Profiler Gene Ontology term analysis of genes that divergent gene overlap with prior studies of *Drosophila melanogaster* and *Drosophila simulans*.