

Sex-Biased Transcriptome Evolution in *Drosophila*

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Abstract

Sex-biased genes are thought to drive phenotypic differences between males and females. The recent availability of high-throughput gene expression data for many related species has led to a burst of investigations into the genomic and evolutionary properties of sex-biased genes. In *Drosophila*, a number of studies have found that X chromosomes are deficient in male-biased genes (demasculinized) and enriched for female-biased genes (feminized) and that male-biased genes evolve faster than female-biased genes. However, studies have yielded vastly different conclusions regarding the numbers of sex-biased genes and forces shaping their evolution. Here, we use RNA-seq data from multiple tissues of *Drosophila melanogaster* and *D. pseudoobscura*, a species with a recently evolved X chromosome, to explore the evolution of sex-biased genes in *Drosophila*. First, we compare several independent metrics for classifying sex-biased genes and find that the overlap of genes identified by different metrics is small, particularly for female-biased genes. Second, we investigate genome-wide expression patterns and uncover evidence of demasculinization and feminization of both ancestral and new X chromosomes, demonstrating that gene content on sex chromosomes evolves rapidly. Third, we examine the evolutionary rates of sex-biased genes and show that male-biased genes evolve much faster than female-biased genes, which evolve at similar rates to unbiased genes. Analysis of gene expression among tissues reveals that this trend may be partially due to pleiotropic effects of female-biased genes, which limits their evolutionary potential. Thus, our findings illustrate the importance of accurately identifying sex-biased genes and provide insight into their evolutionary dynamics in *Drosophila*.

Introduction

Sexual dimorphism, which comprises the morphological and behavioral characteristics that differentiate males and females, is prevalent in the animal kingdom. However, despite often dramatic differences between sexes at the phenotypic level, males and females share nearly identical genomes. Thus, sexual dimorphism is thought to result primarily from differential expression of genes that are present in both sexes, a phenomenon referred to as sex-biased gene expression (Connallon and Knowles 2005; Rinn and Snyder 2005; Ellegren and Parsch 2007).

In *Drosophila*, sex-biased genes are nonrandomly distributed across the genome (Parisi et al. 2003; Sturgill et al. 2007). In particular, *Drosophila* X chromosomes are deficient in male-biased genes (demasculinized) and possibly enriched for female-biased genes (feminized). Three mechanisms have been proposed for this observation. First, inhibition of the expression of X-linked genes during spermatogenesis by meiotic sex chromosome inactivation (MSCI) may result in selection against testis-biased genes on X chromosomes (Lifschytz and Lindsley 1972; Hense et al. 2007; Vibranovski

et al. 2009). Second, dosage compensation, which in *Drosophila* is achieved by overexpression of the male X chromosome, may make it less favorable for male-biased genes to reside on X chromosomes (Vicoso and Charlesworth 2009; Bachtrog et al. 2010). Third, because X chromosomes are preferentially found in females, sexually antagonistic selection may prevent male-biased genes, which are likely beneficial to males and detrimental to females, from accumulating on X chromosomes (Rice 1984; Ellegren and Parsch 2007; Gurbich and Bachtrog 2008). However, despite extensive research in this area, the degree to which each of these mechanisms contributes to the gene content evolution of X chromosomes remains unclear.

Analyses of the evolutionary dynamics of sex-biased genes in *Drosophila* have shown that sex-biased genes evolve more rapidly than unbiased genes, which is expected because sexually dimorphic traits contribute to reproductive success (Ellegren and Parsch 2007). Interestingly, this trend is driven by the evolution of male-biased genes, which have higher turnover (birth/death) rates and evolve faster at the sequence and expression levels than female-biased genes (Meikeljohn

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et al. 2003; Zhang and Parsch 2005; Pröschel et al. 2006; Sawyer et al. 2007; Zhang et al. 2007). Though some evidence shows that these differences are due to stronger positive selection acting on male-biased genes (Zhang and Parsch 2005; Pröschel et al. 2006; Sawyer et al. 2007), another factor may be that female-biased genes are evolutionarily constrained due to greater functional pleiotropy (Parisi et al. 2004; Zhang et al. 2007; Mank et al. 2008; Mank and Ellegren 2009; Meikeljohn and Presgraves 2012).

A major limitation of previous studies is the lack of a clear definition of a sex-biased gene (Ellegren and Parsch 2007; Meisel 2011). In particular, studies have used a wide range of experimental designs, analyses, and thresholds to classify sex-biased genes in *Drosophila*. Nearly all analyses of sex-biased genes in *Drosophila* are based on in-house microarray experiments (Meikeljohn et al. 2003; Ranz et al. 2003; McIntyre et al. 2006; Sturgill et al. 2007; Zhang et al. 2007; Jiang and Machado 2009), in which conditions for raising, collecting, and performing experiments on flies varied widely. Moreover, in the majority of cases, sex-biased genes were identified solely by comparing expression between whole males and whole females (Meikeljohn et al. 2003; Ranz et al. 2003; McIntyre et al. 2006; Sturgill et al. 2007; Zhang et al. 2007; Jiang and Machado 2009), whereas in a few, comparisons between ovary and testis tissue expression were also used (Parisi et al. 2003; Pröschel et al. 2006). To identify sex-biased genes from these comparisons, some investigators used a 2-fold cutoff for male/female expression ratios (Parisi et al. 2003; Pröschel et al. 2006), whereas others used a variety of statistical approaches with different assumptions and significance thresholds (Meikeljohn et al. 2003; Ranz et al. 2003; McIntyre et al. 2006; Sturgill et al. 2007; Jiang and Machado 2009). Because detection of sex-biased gene expression can be affected by differences in experimental conditions (Wyman et al. 2010) and statistical analyses, as well as by variation in biological factors, such as genotype (Jin et al. 2001; Gibson et al. 2004) and age (Jin et al. 2001), it is not surprising that estimates of the fraction of genes with sex-biased expression in *Drosophila* range from 10% to 91%.

We investigated sex-biased transcriptome evolution between *D. melanogaster* and *D. pseudoobscura*, two *Drosophila* species that diverged approximately 21–46 Ma (Beckenbach et al. 1993). In particular, the acquisition of an additional sex chromosome, XR, in *D. pseudoobscura* approximately 8–12 Ma (Richards et al. 2005) enabled us to contrast gene content evolution on sex chromosomes of different ages. Because classification of sex-biased genes is fundamental to studying their evolution, we first compared several independent metrics for identifying male- and female-biased genes. Then, limiting our analysis to genes that were classified as sex biased by multiple metrics, we studied the genomic properties and evolutionary dynamics of sex-biased transcriptome expression in *Drosophila*.

Materials and Methods

Gene Expression Data

Paired-end RNA-seq reads from *D. melanogaster* whole male, whole female, carcass (mixed males and females), male head, female head, testis, accessory gland, and ovary tissues were downloaded from the modENCODE site at <http://www.modencode.org/>. Paired-end RNA-seq reads for whole male, whole female, male carcass, female carcass, testis, accessory gland, and ovary tissues in *D. pseudoobscura* were obtained from Kaiser et al. (2011), and comparable data for male and female head tissues in *D. pseudoobscura* were downloaded from NCBI's sequence read archive (accession numbers SRX016182 and SRX016183).

Bowtie2 (Langmead et al. 2009) was used to align reads to transcript sequences of *D. melanogaster* and *D. pseudoobscura*, using annotation files downloaded from <http://www.flybase.org> (version 3 for both species). Transcript abundances were calculated using eXpress (Roberts and Pachter forthcoming), which outputs read counts and the number of fragments per kilobase of exon per million fragments mapped (FPKM; Trapnell et al. 2010). Quantile normalization of FPKMs was performed using the affy package of Bioconductor in the R software environment (R Development Core Team 2009). By comparing distributions of quantile-normalized FPKMs for exons to those for introns and intergenic sequences (obtained using the procedure outlined for exons), we established cutoffs for expressed transcripts of FPKM = 1 and FPKM = 4 in *D. melanogaster* and *D. pseudoobscura*, respectively. Though different, these cutoffs resulted in similar proportions of expressed genes per tissue in the two species. To enable comparison between the two species, we scaled *D. pseudoobscura* FPKMs to those of *D. melanogaster*.

Identification of Sex-Biased Genes

To identify sex-biased genes in *D. melanogaster* and *D. pseudoobscura*, we applied several independent metrics. First, we simply compared the absolute expression of genes between whole males and whole females, and between testes to ovaries, and obtained genes for which the FPKM was 2-fold higher in one tissue than in the other. In addition to this naïve approach, we also identified genes differentially expressed between whole males and whole females, as well as between testes and ovaries, with three widely used tools: CuffDiff (Trapnell et al. 2010), DESeq (Anders 2010), and edgeR (Robinson et al. 2009). CuffDiff takes a nonparametric, annotation-guided approach to estimate the means and variances of transcripts' FPKMs in different conditions, using Student's *t*-tests to identify differentially expressed transcripts (Trapnell et al. 2010). In contrast, DESeq and edgeR estimate the means and variances of raw read counts under a negative binomial distribution and use exact tests to identify differentially expressed transcripts. The main difference between

DESeq and edgeR is that they use different statistical approaches to estimate variance (Robinson et al. 2009; Anders 2010).

As a last metric, we obtained all sex tissue-specific genes (those primarily expressed in testes, accessory glands, or ovaries) by using the following formula to calculate the tissue specificity index, τ , for each gene:

$$\tau = \frac{\sum_{i=1}^N 1 - \frac{\log E_i}{\log E_{\max}}}{N - 1},$$

where N is the number of tissues, E_i is the expression in tissue i , and E_{\max} is the maximum expression of the gene in all tissues (Yanai et al. 2005; Larracuente et al. 2008). τ ranges from 0 to 1, with larger τ values indicating greater tissue specificity. We applied a cutoff of $\tau \geq 0.9$ to obtain highly tissue-specific genes (Larracuente et al. 2008). Because genes that are expressed in multiple tissues are more likely to have complex functions than those expressed in a single tissue (McShea 2000), τ can also be used to approximate the pleiotropy of genes (Mank et al. 2008; Mank and Ellegren 2009; Meisel 2011; Meikeljohn and Presgraves 2012). Therefore, in our analyses, we assumed that broadly expressed genes (those with low τ values) are more pleiotropic than narrowly expressed genes (those with high τ values).

Evolution of Sex-Biased Genes

We obtained orthologs for sex-biased and unbiased genes from *Drosophila* ortholog tables downloaded from <http://www.flybase.org>, which we supplemented with genes from reciprocal best Basic Local Alignment Search Tool (BLAST, Altschul et al. 1990) and BLAST-Like Alignment Tool (BLAT, Kent 2002) searches. Our turnover analysis was conservative in that, even if a one-to-one ortholog could not be identified for a gene in the sister species, it was not considered to be absent unless it had no potential orthologs in the genome of that species. Our analysis of expression divergence was also conservative; we only considered a gene as having gained/lost its sex-biased expression if it was identified as sex biased in one species by multiple metrics ($n = 2$ or $n = 4$; see main text for details) and not identified as sex biased by any metrics in the sister species.

We downloaded *D. melanogaster* and *D. pseudoobscura* CDS sequences from <http://www.flybase.org> and aligned orthologous genes with MACSE (Ranwez et al. 2011), which performs in-frame alignments of protein-coding sequences. PAML (Yang 2007) was used to estimate the pairwise K_a/K_s from these alignments.

Statistical Analyses

We used Wilcoxon tests to compare the overall expression level, sex-biased expression, and tissue specificity among chromosomes, as well as to compare the K_a/K_s of unbiased,

male-biased, and female-biased genes. The expected number of male-/female-biased genes on a particular chromosome was determined by multiplying the total number of male-/female-biased genes in the genome by the proportion of all genes on that chromosome. χ^2 tests were used to determine whether the observed and expected numbers of male- and female-biased genes on sex chromosomes were significantly different. All statistical analyses were performed in the R software environment (R Development Core Team 2009).

Results

Identification of Sex-Biased Genes in *D. melanogaster* and *D. pseudoobscura*

We applied nine independent metrics to identify genes with sex-biased expression in *D. melanogaster* and *D. pseudoobscura*. First, we obtained genes differentially expressed in whole males and whole females, as well as in testes and ovaries, by four analyses: comparison of absolute expression levels between male and female tissues (2-fold cutoff), CuffDiff (Trapnell et al. 2010), DESeq (Anders 2010), and edgeR (Robinson et al. 2009). Additionally, we ascertained genes expressed primarily in the sex tissues of males (testis and accessory gland) or females (ovary) by calculating breadths of gene expression (see Materials and Methods for details).

Depending on the metric used, 6.9–28.1% of genes in *D. melanogaster* and 6.6–24.1% of genes in *D. pseudoobscura* are classified as male biased, and 0.3–28.5% of genes in *D. melanogaster* and 0.2–35.9% of genes in *D. pseudoobscura* are classified as female biased (table 1; see [supplementary tables S1–S4, Supplementary Material](#) online, for lists of genes and metrics). In total, approximately 42.8% of genes in *D. melanogaster* and 31% of genes in *D. pseudoobscura* are classified as male biased by at least one metric, and 34.8% of genes in *D. melanogaster* and 46% of genes in *D. pseudoobscura* are classified as female biased by at least one metric. However, the overlap of genes satisfying multiple definitions is small, particularly for female-biased genes (fig. 1 and [supplementary tables S1–S4, Supplementary Material](#) online). There are 339 and 493 genes identified as male biased by all metrics in *D. melanogaster* and *D. pseudoobscura*, respectively, only 16 genes identified as female biased by all metrics in *D. melanogaster*, and not a single gene identified as female biased by all metrics in *D. pseudoobscura*. Thus, male-biased genes are identified more robustly than female-biased genes.

In total, more than 75% of genes in each species were classified as sex biased by at least one metric. To determine whether sets of genes identified by different metrics have unique properties, we compared the proportions of X-linked male- and female-biased genes among metrics. This analysis revealed that genes identified by different metrics tend to have different genomic distributions ([supplementary table S5, Supplementary Material](#) online). Hence, rather

Table 1

Sex-Biased Genes Identified by Various Metrics

	Male-Biased Genes		Female-Biased Genes	
	<i>D. melanogaster</i>	<i>D. pseudoobscura</i>	<i>D. melanogaster</i>	<i>D. pseudoobscura</i>
Body, 2-fold	3,871	3,148	3,512	5,737
Body, CuffDiff	1,081	1,980	225	188
Body, DESeq	948	1,054	39	24
Body, edgeR	1,635	1,964	89	55
Gonads, 2-fold	3,864	3,851	3,927	4,826
Gonads, CuffDiff	3,713	2,201	1,559	295
Gonads, DESeq	1,690	1,348	37	30
Gonads, edgeR	2,450	2,664	105	96
Sex tissue-specific	2,220	2,412	413	408

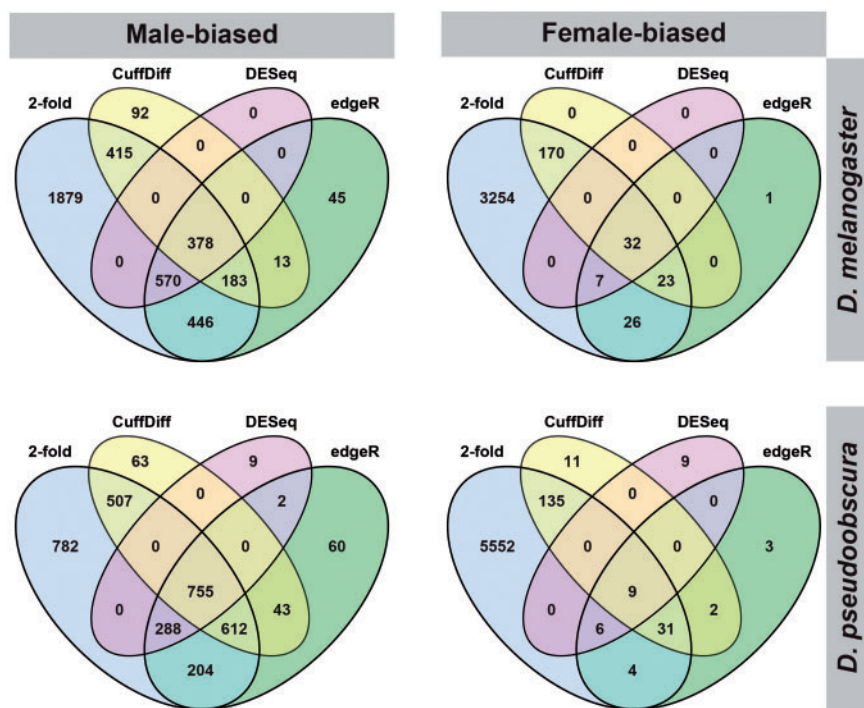


FIG. 1.—Comparison of four metrics for classifying sex-biased genes. Venn diagrams of numbers of male-biased (left) and female-biased (right) genes identified by four comparisons of whole male and whole female tissues in *D. melanogaster* (top) and *D. pseudoobscura* (bottom).

than choosing a specific metric(s), we defined sex-biased genes as those identified by any combination of at least n metrics. To investigate the relationship between n and degree of sex-biased gene expression, we plotted n against whole male/whole female expression (fig. 2). A key observation is that variance in sex-biased expression increases with n , illustrating the strong positive correlation between the number of metrics used and the degree of sex-biased effect of genes identified. Moreover, as n increases, the distance between genes classified as male- and female-biased also increases. As expected, n for male-biased genes is positively

correlated to whole male/whole female expression ($\rho = 0.80$ for *D. melanogaster* and $\rho = 0.83$ for *D. pseudoobscura*), whereas n for female-biased genes is negatively correlated to whole male/whole female expression ($\rho = -0.60$ for *D. melanogaster* and $\rho = -0.22$ for *D. pseudoobscura*). In general, the strengths of these correlations are consistent with the robustness of sex-biased gene identification (fig. 1 and [supplementary tables S1–S4, Supplementary Material online](#)).

In choosing n for defining sex-biased genes, we considered that requiring support from too few metrics would result in

many false positives (Type I errors), whereas requiring support from too many metrics would produce many false negatives (Type II errors). For this reason, we compared the numbers of overlapping and nonoverlapping sex-biased genes for different n and selected two separate thresholds of $n=2$ and $n=4$. Genes that were classified as sex biased by at least any of two or four metrics were assigned to the $n=2$ and $n=4$ categories, respectively. Thus, $n=4$ is a subset of $n=2$. For $n=2$, there are 4,042 male- and 3,038 female-biased genes in *D. melanogaster*, and 3,512 male- and 3,123 female-biased genes in *D. pseudoobscura*. For $n=4$, there are 2,281 male- and 331 female-biased genes in *D. melanogaster*, and 2,463 male- and 136 female-biased genes in *D. pseudoobscura*.

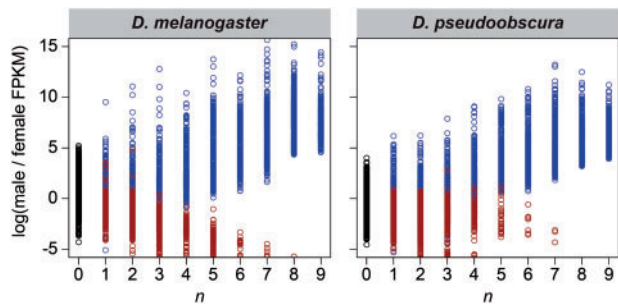


FIG. 2.—Relationship between n and sex-biased gene expression. Plots show correlations between the number of metrics (n) that identify genes as male biased (blue) or female biased (red) and the whole male/whole female expression ratios of those genes in *D. melanogaster* (left) and *D. pseudoobscura* (right).

Chromosomal Distribution of Sex-Biased Genes in *D. melanogaster* and *D. pseudoobscura*

Studies in *Drosophila* have revealed that there is an under-representation of male-biased genes (demasculinization) and over-representation of female-biased genes (feminization) on X chromosomes (Parisi et al. 2003; Sturgill et al. 2007). To investigate the extent of demasculinization and feminization in our data, we used four separate measures. First, we compared the absolute expression between genes on X chromosomes and autosomes in different tissues (fig. 3). Consistent with previous studies, we found that expression of genes in whole males, testes, and accessory glands is lower on the X chromosome in *D. melanogaster* and on both the XL and XR chromosomes in *D. pseudoobscura*, than on autosomes in either species, supporting demasculinization of X chromosomes. Additionally, the expression of genes in whole females and ovaries is significantly higher on the X chromosome than on autosomes in *D. melanogaster*, supporting feminization of the *D. melanogaster* X, though there is no evidence for feminization of either XL or XR in *D. pseudoobscura*.

Second, we compared the contribution of sex-specific tissue to whole body expression (testis/whole male, accessory gland/whole male, and ovary/whole female expression) on X chromosomes and autosomes in *D. melanogaster* and *D. pseudoobscura* (fig. 4). In both species, we found evidence for demasculinization of X chromosomes when comparing testis/whole male contributions and feminization when comparing ovary/whole female contributions. Surprisingly, however, accessory gland tissue contributions

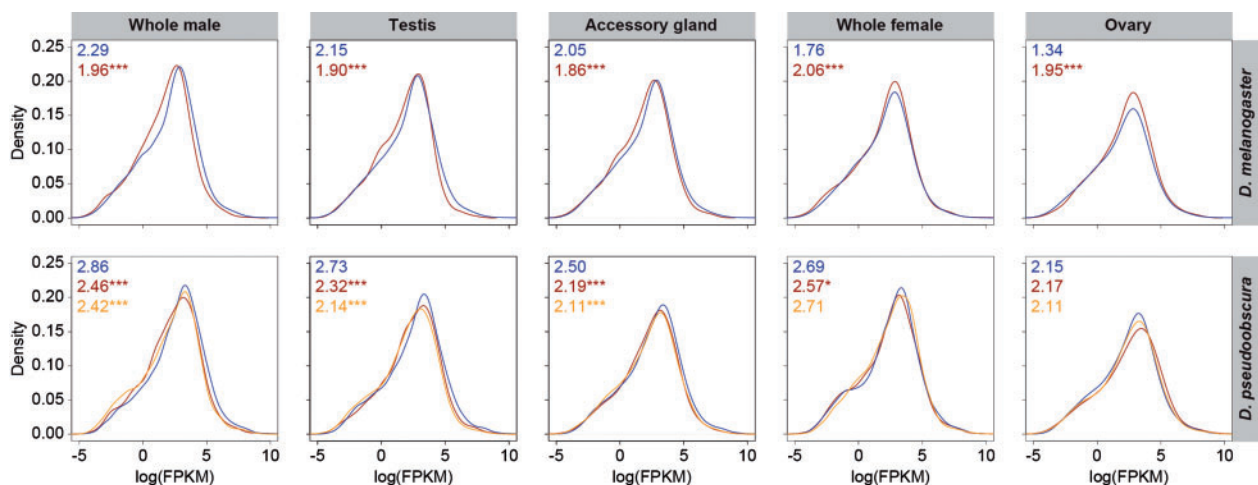


FIG. 3.—Absolute expression on X chromosomes and autosomes in different tissues. Density plots of absolute expression for X chromosomes and autosomes in different tissues of *D. melanogaster* (top) and *D. pseudoobscura* (bottom). Each panel corresponds to a different tissue, with autosomal densities depicted in blue, ancestral X chromosome (*D. melanogaster* X and *D. pseudoobscura* XL) densities depicted in red, and *D. pseudoobscura* XR densities depicted in orange. Medians are given in the top left corner of each panel, with asterisks corresponding to $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

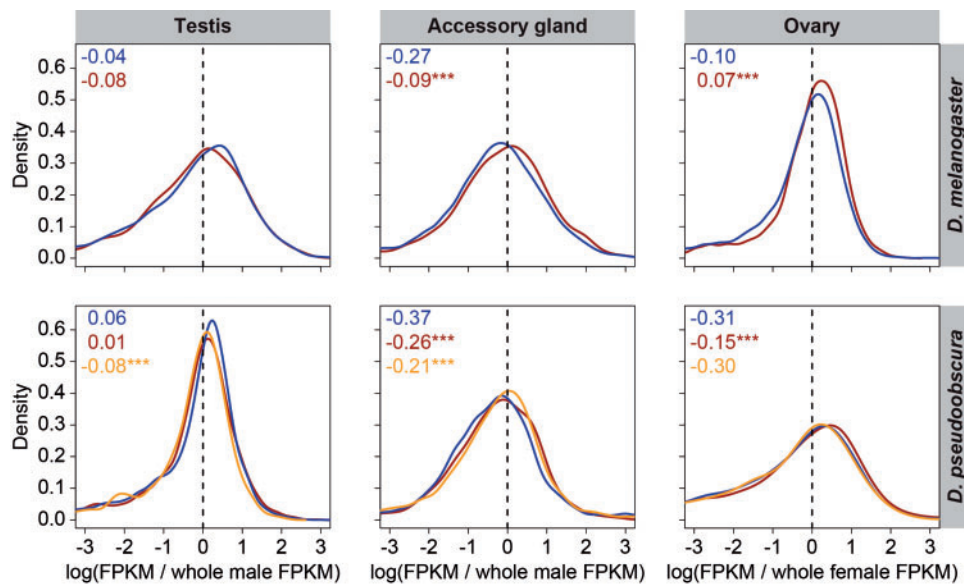


FIG. 4.—Contributions of sex-specific tissue expression on X chromosomes and autosomes. Density plots of sex-specific tissue/whole body expression in *D. melanogaster* (top) and *D. pseudoobscura* (bottom). Each panel corresponds to a different sex-specific tissue, with autosomal densities depicted in blue, ancestral X chromosome (*D. melanogaster* X and *D. pseudoobscura* XL) densities depicted in red, and *D. pseudoobscura* XR densities depicted in orange. Medians are given in the top left corner of each panel, with asterisks corresponding to $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

were significantly higher on the X chromosome in *D. melanogaster* and on both XL and XR in *D. pseudoobscura* than on autosomes in either species, providing evidence for masculinization of *Drosophila* X chromosomes.

Third, we compared sex-biased expression of whole body (whole male/whole female) and gonad (testis/ovary) tissues on X chromosomes and autosomes (fig. 5). Consistent with previous studies, we found that whole male/whole female and testis/ovary expression ratios are significantly higher on autosomes than on X chromosomes in *D. melanogaster* and *D. pseudoobscura*, providing evidence for demasculinization and/or feminization of the X chromosomes in both species.

As a final approach, we compared observed and expected numbers of male- and female-biased genes on *D. melanogaster* and *D. pseudoobscura* X chromosomes (fig. 6). We calculated expected numbers by assuming that sex-biased genes are randomly distributed across chromosomes and that their representation on a particular chromosome is proportional to the actual number of genes on that chromosome. For $n=2$, the observed numbers of male-biased genes on *D. melanogaster* X and on *D. pseudoobscura* XL and XR are significantly lower than expected, and the observed numbers of female-biased genes on *D. melanogaster* X and on *D. pseudoobscura* XL and XR are significantly higher than expected, supporting demasculinization and feminization of all *Drosophila* X chromosomes. However, for $n=4$, demasculinization is not observed for *D. pseudoobscura* XL, and the significance

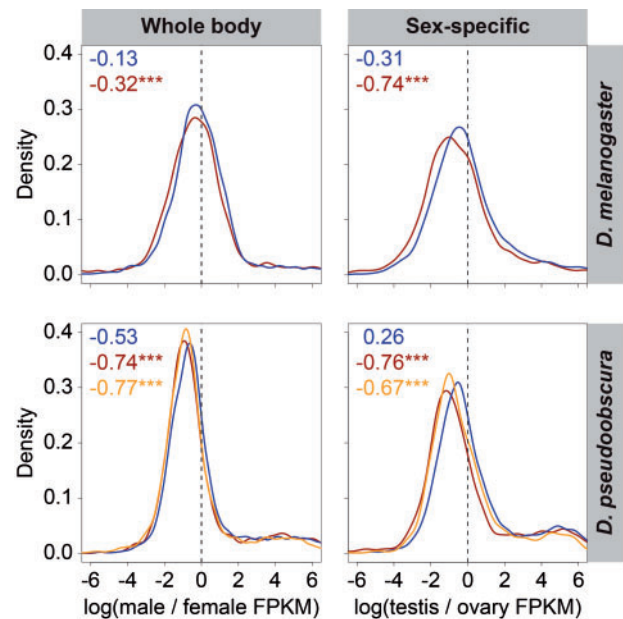


FIG. 5.—Sex-biased expression on X chromosomes and autosomes. Density plots of whole male/whole female (left) and testis/ovary (right) expression ratios in *D. melanogaster* (top) and *D. pseudoobscura* (bottom). Autosomal densities are depicted in blue, ancestral X chromosome (*D. melanogaster* X and *D. pseudoobscura* XL) densities are depicted in red, and *D. pseudoobscura* XR densities are depicted in orange. Vertical dashed lines indicate equal male and female expression. Medians are given in the top left corner of each panel, with asterisks corresponding to $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

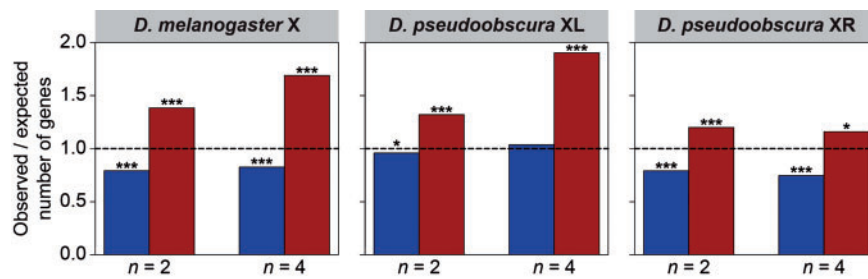


FIG. 6.—Representation of sex-biased genes on *Drosophila* X chromosomes. Proportions of observed/expected numbers of male-biased (blue) and female-biased (red) genes on *D. melanogaster* X, *D. pseudoobscura* XL, and *D. pseudoobscura* XR. The number of metrics (n) used in the classification of sex-biased genes for each analysis is indicated on the x axis. Horizontal dashed lines indicate equal observed and expected numbers of genes, and asterisks indicate $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

of feminization of *D. pseudoobscura* XR is weaker than for $n = 2$.

Though most approaches revealed a general trend of demasculinization and feminization of *Drosophila* X chromosomes, aside from more expressed X-linked genes in *D. melanogaster* ovaries, the total numbers of expressed genes in different tissues do not differ between the X chromosomes and autosomes. Therefore, demasculinization does not simply result from a lower proportion of X-linked genes expressed in testis, as might be expected if X chromosomes are transcriptionally silenced during spermatogenesis or if sexually antagonistic selection removes individual male-biased genes. Rather, lower male-biased expression appears to be a property of most genes residing on X chromosomes.

Evolution of Sex-Biased Genes between *D. melanogaster* and *D. pseudoobscura*

To study the evolution of sex-biased genes, we obtained pairs of orthologous genes in *D. melanogaster* and *D. pseudoobscura* and calculated the turnover, expression divergence, and sequence evolutionary rates of genes identified as male and female biased in the two species. Turnover of a sex-biased gene was inferred when that gene did not have an ortholog in the sister species (see Materials and Methods for details), and the rate of turnover was estimated by dividing the number of such genes by the total number of sex-biased genes. Comparison of sex-biased gene turnover rates confirmed previous findings that, in *D. melanogaster* and *D. pseudoobscura*, male-biased genes have much higher turnover rates than female-biased genes (for $n = 2$ and $n = 4$). Although nearly all female-biased genes are present in both species, approximately 19.6–37.6% of male-biased genes in either *D. melanogaster* or *D. pseudoobscura* lack orthologs in the other species (fig. 7A).

Expression divergence was inferred when a gene was classified as male or female biased ($n = 2$ or $n = 4$) in one species and not classified as male/female biased by any metrics in the sister species. The rate of evolution by expression divergence was estimated by dividing the number of genes with

expression divergence by the total number of sex-biased genes containing orthologs in the sister species. Comparison of the rates between male- and female-biased genes revealed opposite patterns in *D. melanogaster* and *D. pseudoobscura* (fig. 7B).

In *D. melanogaster*, higher proportions of male- than female-biased genes do not have the same sex bias in *D. pseudoobscura*. On the other hand, in *D. pseudoobscura*, more female- than male-biased genes do not have the same sex bias in *D. melanogaster*. Interestingly, this difference seems to be due to contrasting rates of switches between male- and female-biased states in the two species (table 2). In particular, from *D. melanogaster* to *D. pseudoobscura*, male-to-female-biased switches occur 4.2–5.6 times faster than female-to-male-biased switches. In contrast, from *D. pseudoobscura* to *D. melanogaster*, female-to-male-biased switches occur 2.7–4.7 times faster than male-to-female-biased switches. Thus, from *D. melanogaster* to *D. pseudoobscura*, changes in sex-biased states tend to be in the male-to-female direction, whereas the opposite pattern is observed from *D. pseudoobscura* to *D. melanogaster*.

To study the sequence divergence of sex-biased genes, we calculated sequence evolutionary rates (K_A/K_S) of unbiased, male-biased, and female-biased genes in *D. melanogaster* and *D. pseudoobscura*. In both species, for $n = 2$ and $n = 4$, sex-biased genes evolve much faster than unbiased genes at the DNA sequence level ($P < 0.001$). Comparison of male- and female-biased genes revealed that male-biased genes evolve significantly faster than female-biased genes (fig. 7C, $P < 0.001$), which evolve at similar rates as unbiased genes. Thus, we confirmed previous findings that faster evolution of sex-biased genes is primarily due to the rapid evolution of male-biased genes.

Faster evolution of male-biased genes is often attributed to stronger positive selection acting on male- than on female-biased genes (Zhang and Parsch 2005; Pröschel et al. 2006; Sawyer et al. 2007). However, another possible factor is that female-biased genes are more pleiotropic, restricting their adaptive potential (Parisi et al. 2004; Zhang et al. 2007).

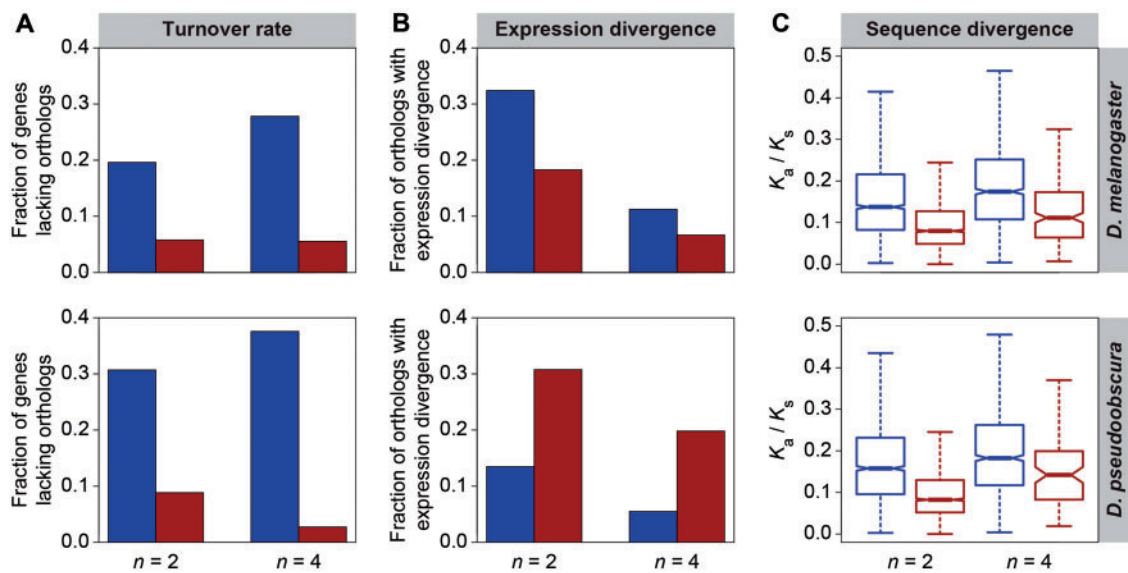


FIG. 7.—Evolution of sex-biased genes in *Drosophila*. Rates of *D. melanogaster* (top) and *D. pseudoobscura* (bottom) male-biased (blue) and female-biased (red) gene turnover (A), expression divergence (B), and sequence divergence (C). The number of metrics (n) used in the classification of sex-biased genes for each analysis is indicated on the x axis.

Table 2
Rates of Switches between Male- and Female-Biased Gene States

	$n = 2$		$n = 4$	
	M → F	F → M	M → F	F → M
<i>D. melanogaster</i>	0.187	0.045	0.067	0.012
<i>D. pseudoobscura</i>	0.051	0.139	0.014	0.066

This hypothesis is supported by the observation that most male-biased expression in *D. melanogaster* results from genes expressed in a sex-specific tissue(s), whereas female-biased genes tend to be broadly expressed (Parisi et al. 2004; Sturgill et al. 2007; Meikeljohn and Presgraves 2012). Examination of the contribution of individual tissues to sex-biased expression revealed that only testis and accessory gland expression levels are positively correlated to sex-biased expression ($P < 0.05$), illustrating the influence of male sex tissue-specific expression to overall male-biased gene expression (supplementary fig. S1, Supplementary Material online). To further investigate the pleiotropy hypothesis, we compared tissue specificity (τ) to whole male/whole female expression ratios in *D. melanogaster* and *D. pseudoobscura* (fig. 8). This analysis shows that, in both species, female-biased genes tend to have smaller τ values than male-biased genes, supporting the hypothesis that female-biased genes exhibit greater pleiotropy. Thus, female-biased genes are expected to be under greater selective constraint than male-biased genes, likely contributing to their slower evolution.

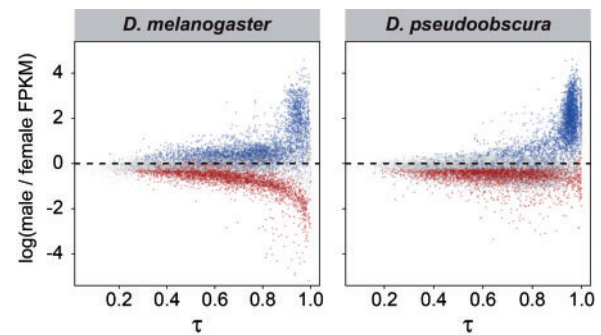


FIG. 8.—Tissue specificity of sex-biased genes. Correlations between whole male/whole female expression ratios and τ in *D. melanogaster* (left) and *D. pseudoobscura* (right). Unbiased genes are represented by gray dots, male-biased genes by blue dots, and female-biased genes by red dots ($n = 2$). Horizontal dashed lines indicate equal male and female expression.

To determine whether gene turnover or switches in sex-biased expression contribute to demasculinization of X chromosomes, we compared the turnover and expression evolutionary rates of different Muller elements, which represent orthologous chromosomal segments, in *D. melanogaster* and *D. pseudoobscura* (fig. 9). We found that, in each species, male-biased genes on Muller-A, the ancestral X chromosome, are least likely to have orthologs in the other species ($P < 0.01$). Furthermore, genes that are male biased in *D. melanogaster* are most likely to show female-biased expression in *D. pseudoobscura*, and genes that are female biased

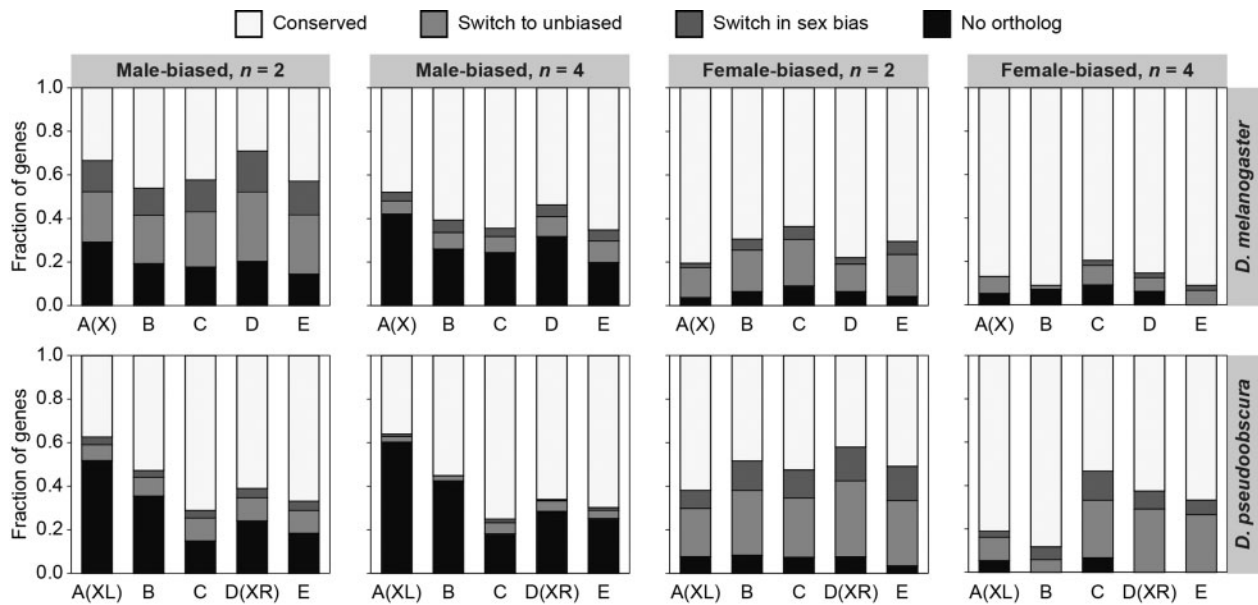


Fig. 9.—Turnover and expression evolution of sex-biased genes among chromosomes. Proportions of male- and female-biased genes on each Muller element of *D. melanogaster* (top) and *D. pseudoobscura* (bottom) that, in the other species, have conserved expression, are unbiased, have opposite sex-biased expression, or do not have orthologs.

in *D. pseudoobscura* are most likely to display male-biased expression in *D. melanogaster*, when they are located on Muller-D, which is autosomal chromosome 3L in *D. melanogaster* and chromosome XR in *D. pseudoobscura*. Thus, both turnover in gene content and switches in expression bias contribute to the deficiency of male-biased genes on *Drosophila* X chromosomes.

Discussion

Classification of Sex-Biased Genes Is Complex

The continuous nature of sex-biased gene expression makes it difficult to consistently identify genes with the greatest sex-biased effects, complicating meaningful comparisons among taxa and hindering our understanding of sex-biased transcriptome evolution. In this study, we compared nine independent metrics for identifying sex-biased genes in *D. melanogaster* and *D. pseudoobscura*. We found that different metrics yielded vastly different numbers and sets of sex-biased genes in both species, often with little overlap in the genes identified across metrics. However, this is not too surprising, given that the metrics are based on different biological and/or statistical models and have different assumptions, statistical methodologies, and false positive/negative rates. Because analysis of the sex-biased genes identified by different metrics did not reveal an optimal metric or set of metrics, we chose to define a sex-biased gene based on its support by a minimum number of metrics, *n*. We compared numbers of sex-biased genes for different *n*, and, to minimize

Type I and Type II errors, we chose two separate cutoffs for the number of metrics supporting each gene: *n* = 2 and *n* = 4. Because male-biased genes are more robustly identified across metrics, each of these cutoffs yielded more male- than female-biased genes, with *n* = 2 resulting in over ten times more genes classified as female biased than *n* = 4. There are two possible reasons for this effect. First, there may be few female-biased genes, and, therefore, higher stringency (*n* = 4) enhances the identification of female-biased genes by eliminating Type I errors. On the other hand, female-biased genes may be more difficult to identify, possibly because they are pleiotropic or expressed at lower levels than male-biased genes, leading to a general lack of female-biased genes among metrics. In this case, Type II errors are more problematic, perhaps making *n* = 2 a better cutoff for female-biased genes. Thus, there was a twofold benefit to using both cutoffs in our analysis. It enabled us to compare conclusions reached by using different cutoffs, as well as to uncover well-supported patterns in our data.

Drosophila X Chromosomes Are Demasculinized and Feminized

Analysis of chromosomal gene content in *Drosophila* by four separate approaches revealed a general deficiency of male-biased genes and enrichment of female-biased genes on *Drosophila* X chromosomes. The exception to this result is accessory gland/whole male expression, which instead supports masculinization of X chromosomes (fig. 4). Interestingly, demasculinization of X chromosomes is observed from overall

accessory gland expression patterns (fig. 3). To investigate this effect, we compared accessory gland/male carcass tissue expression on *D. pseudoobscura* X chromosomes and autosomes. Unfortunately, because of the lack of male carcass expression data for *D. melanogaster*, we were unable to perform this analysis in both species. In *D. pseudoobscura*, accessory gland/male carcass tissue expression was higher on autosomes than on X chromosomes, providing support for demasculinization of X chromosomes. Because male carcass tissue includes all individual tissues found in whole males, with the exception of sex-specific (testis and accessory gland) tissues, this result suggests that the masculinization pattern observed from accessory gland/whole male comparisons is driven by lower testis expression on X chromosomes relative to autosomes. Thus, this pattern does not appear to be biologically interesting, and the general finding is that X chromosomes are demasculinized.

Though *D. pseudoobscura* XR only became a sex chromosome 8–12 Ma (Richards et al. 2005), it already shows evidence of demasculinization and feminization, revealing that gene content evolution occurs rapidly after acquisition of sex chromosomes. Interestingly, in *D. pseudoobscura*, demasculinization is stronger on XR than on XL, the ancestral X chromosome, and feminization is stronger on XL than on XR. Because XR is a newer sex chromosome, this effect may be caused by demasculinization proceeding at a faster rate than feminization, presumably because male-biased genes evolve faster than female-biased genes.

MSCI (Lifschytz and Lindsley 1972; Hense et al. 2007; Vrbancovski et al. 2009), dosage compensation (Bachtrog et al. 2010), and sexually antagonistic selection (Rice 1984; Ellegren and Parsch 2003; Gurbich and Bachtrog 2008) have each been proposed to explain demasculinization of X chromosomes. Under the MSCI hypothesis, we would expect to observe demasculinization only in testis and whole male tissues, and we would also expect there to be fewer X-linked genes expressed in testes. Although we do observe demasculinization in testes and whole males, we also observe demasculinization in accessory glands when we consider absolute expression of genes. Moreover, there is no significant difference in the proportions of testis-expressed genes on X chromosomes and autosomes. These observations, together with the recent finding that MSCI may not operate in *Drosophila* (Meikeljohn et al. 2011), suggest that MSCI is not responsible for demasculinization of *Drosophila* X chromosomes. In contrast, dosage compensation could cause demasculinization patterns in testes and whole males, as well as in accessory glands, for two separate reasons. Recent data suggest that testes in *Drosophila* completely lack dosage compensation (Meikeljohn et al. 2011), and generally lower expression levels of X-linked genes in testes are consistent with that finding (Meikeljohn and Presgraves 2012). In accessory glands, the dosage compensation machinery could interfere with the evolution of male-biased gene expression (Parisi

et al. 2004). Additionally, dosage compensation would not reduce the proportion of genes expressed in either testes or accessory glands on the X chromosome, which is consistent with our findings. Sexually antagonistic selection, on the other hand, operates on a gene-by-gene basis. Thus, we would expect to observe fewer testis-expressed and more ovary-expressed genes on X chromosomes, which is not supported by our data. Instead, lower expression appears to be a general property of genes residing on *Drosophila* X chromosomes. Thus, our data support dosage compensation as a major mechanism driving demasculinization of *Drosophila* X chromosomes. However, it is important to note that many independent forces likely contribute to sex chromosome evolution, and disentangling these forces may be impossible from a chromosome-wide perspective.

Male-Biased Genes Evolve Faster than Female-Biased Genes

Consistent with previous studies (Meikeljohn et al. 2003; Zhang and Parsch 2005; Pröschel et al. 2006; Sawyer et al. 2007; Zhang et al. 2007), our analyses revealed that male-biased genes have higher turnover and sequence evolutionary rates than female-biased genes in *D. melanogaster* and *D. pseudoobscura*. We also found that evolution by expression divergence is faster for male-biased genes in *D. melanogaster* and for female-biased genes in *D. pseudoobscura*. Further analysis revealed that this contrast is likely driven by species-specific differences in rates of switches between male-to-female-biased and female-to-male-biased expression. In particular, male-biased genes in *D. melanogaster* are more likely to display female-biased expression in *D. pseudoobscura*, and female-biased genes in *D. pseudoobscura* are more likely to display male-biased expression in *D. melanogaster*. The highest switch rates in *D. pseudoobscura* occur among female-biased genes on chromosome XR, which is a recently acquired sex chromosome in *D. pseudoobscura* and is orthologous to autosomal chromosome 3L in *D. melanogaster*. The most likely explanation for this pattern is that many genes residing on the ancestral autosomal chromosome switched from male- to female biased after it became a sex chromosome in the *D. pseudoobscura* lineage. Thus, differences in switch rates may contribute to demasculinization and feminization of *D. pseudoobscura* chromosome XR.

The faster evolution of male-biased genes is generally attributed to stronger positive selection acting on male-biased relative to female-biased genes (Zhang and Parsch 2005; Pröschel et al. 2006; Sawyer et al. 2007), which is supported by the higher K_a/K_s rates of male-biased genes. However, another factor that may contribute to this pattern is functional pleiotropy of female-biased genes, which would limit their evolutionary potential (Parisi et al. 2004; Zhang et al. 2007). The similarity of K_a/K_s in female-biased and unbiased genes is consistent with this hypothesis. Moreover, comparison of

tissue specificities of male- and female-biased genes revealed that female-biased genes are generally more broadly expressed than male-biased genes. Thus, our findings support the hypothesis that pleiotropy of female-biased genes constrains their evolution, contributing to their slower evolution relative to male-biased genes.

Supplementary Material

Supplementary figure S1 and tables S1–S5 are available at *Genome Biology and Evolution* online (<http://www.gbe.oxfordjournals.org/>).

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