Little Evidence for Demasculinization of the *Drosophila X* Chromosome among Genes Expressed in the Male Germline

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Abstract

Male-biased genes—those expressed at higher levels in males than in females—are underrepresented on the X chromosome of *Drosophila melanogaster*. Several evolutionary models have been posited to explain this so-called demasculinization of the X. Here, we show that the apparent paucity of male-biased genes on the X chromosome is attributable to global X-autosome differences in expression in *Drosophila* testes, owing to a lack of sex chromosome dosage compensation in the male germline, but not to any difference in the density of testis-specific or testis-biased genes on the X chromosome. First, using genome-wide gene expression data from 20 tissues, we find no evidence that genes with testis-specific expression are underrepresented on the X chromosome. Second, using contrasts in gene expression profiles among pairs of tissues, we recover a statistical underrepresentation of testis-biased genes on the X but find that the pattern largely disappears once we account for the lack of dosage compensation in the *Drosophila* male germline. Third, we find that computationally "demasculinizing" the autosomes is not sufficient to produce an expression profile similar to that of the X chromosome in the testes. Our findings thus show that the lack of sex chromosome dosage compensation in *Drosophila* testes can explain the apparent signal of demasculinization on the X, whereas evolutionary demasculinization of the X cannot explain its overall reduced expression in the testes.

Key words: *Drosophila*, X chromosome, sex-biased gene expression.

Introduction

More than ~4,000 genes in the *Drosophila* genome exhibit sex-biased gene expression, having higher transcript levels in one sex than the other (Gnad and Parsch 2006). In *Drosophila melanogaster* (Parisi et al. 2003, 2004) and closely related species (Ranz et al. 2003; Sturgill et al. 2007), genes with male-biased expression are overwhelmingly testes expressed and, curiously, underrepresented on the X chromosome. To explain the seemingly nonrandom genomic distribution of male-biased genes, two kinds of evolutionary models have been posited. First, a "demasculinized" X chromosome may reflect a history of sexually antagonistic natural selection (Parisi et al. 2003; Wu and Xu 2003; Sturgill et al. 2007). As the X chromosome spends two-thirds of its ancestry in females and only one-third in males, partially dominant genetic variants that are beneficial to males but deleterious to females can increase in populations when rare more readily on the autosomes than on the X (Rice 1984). Second, in many taxa, the X chromosome experiences meiotic sex chromosome inactivation (MSCI), the facultative heterochromatinization and transcriptional silencing of the sex chromosomes prior to the autosomes during early meiosis I (Lifschytz 1972; Turner 2007; Namekawa and Lee 2009). By restricting X-linked transcription in the germline, MSCI could in principle compromise optimal gene expression levels, thereby favoring the evolution of compensatory gene duplications and/or transpositions to the autosomes (Betran et al. 2002). Consistent with these models, the *Drosophila* genome harbors an excess of duplicated retrogenes on the autosomes that originated from parent copies on the X chromosome (Betran et al. 2002; Vibranovski et al. 2009). Both of these evolutionary models are based on the premise that the X chromosome is, for one reason or another, an unfavorable location for genes with male-specific functions.

These models describe evolved differences in the gene content of the X chromosome and the autosomes. However, the data indicating a demasculinized X come exclusively from gene expression assays (microarrays and RNA-seq) that

compare relative transcript levels in males versus females or in testes versus ovaries. Previous studies reported that average relative expression from the X and the autosomes is statistically indistinguishable in the testes (Parisi et al. 2003; Gupta et al. 2006; Sturgill et al. 2007), and therefore the deficit of male-biased genes was inferred to result from a distinct gene content on the X. However, reanalysis of multiple gene expression datasets using both microarrays and RNA-seq shows that median expression levels of X-linked genes when assayed in whole testes are in fact approximately 1.5-fold lower than those of autosomal genes (Meiklejohn et al. 2011). It is therefore possible that the apparent paucity of genes with male-biased expression on the *Drosophila* X chromosome could result from differences in global expression levels between the X and the autosomes in testes *versus* ovaries, with little or no differences in evolved gene content.

Two competing models have been proposed to explain the lower overall X-linked gene expression levels in *Drosophila* testes. The first is that MSCI occurs in *Drosophila* as it does in mammals and *C. elegans* (Namekawa and Lee 2009), leading to reduced expression from the X chromosome versus the autosomes in the testes (Vibranovski et al. 2009). Under this hypothesis, the lower X chromosome expression in whole testis is the read-out of a mixed population of cells, including those in which the X is expressed at levels equal to the autosomes (implying X chromosome dosage compensation) and a small subset of cells, presumably early meiotic spermatocytes, in which the X is transcriptionally inactive (Vibranovski et al. 2009). However, aside from 1.5-fold lower median expression, gene expression assays show little to no evidence of stage-specific meiotic inactivation of the X (Meiklejohn et al. 2011; Mikhaylova and Nurminsky 2011), and there is no alternate, direct support for MSCI in *Drosophila*. The second model is that the X-autosome difference in expression results from a lack of sex chromosome dosage compensation in the *Drosophila* male germline (Meiklejohn et al. 2011). In the male soma of *Drosophila*, the sex chromosome dosage compensation complex (DCC) comprises at least five proteins and two RNAs that together facilitate hypertranscription of the single X chromosome (Gelbart and Kuroda 2009). In the male germline, however, the DCC is absent (Rastelli and Kuroda 1998), and the magnitude of the observed X-autosome expression difference in the testis is consistent with that predicted to result from haploid expression in *Drosophila* (Meiklejohn et al. 2011).

In this article, we revisit the observation of X chromosome demasculinization and examine the relationship between gene content and gene expression of the *Drosophila* X chromosome in the male germline. Specifically, we test the hypothesis that the apparent paucity of genes with male-biased expression on the *Drosophila* X chromosome is driven primarily by global differences in gene expression levels between the X and the autosomes, rather than differences in gene content. Our results show that global expression levels, not evolved gene content, cause the apparent underrepresentation of testis-biased genes on the X—with respect to the male germline, the *Drosophila* X chromosome is not demasculinized.

Materials and Methods

We compiled microarray assays of gene expression in 20 tissues and organs dissected from wild-type larval and adult *D. melanogaster* (FlyAtlas—Chintapalli et al. 2007; NCBI Gene Expression Omnibus accession GSE 7763), 11 tissues dissected from adult *Anopheles gambiae* (Baker et al. 2011; GSE 21689), and *D. melanogaster bag-of-marbles* (*bam*) mutant testis (Chen et al. 2011; GSE 28728). RNA-seq data (Gan et al. 2010) were analyzed as previously described (Meiklejohn et al. 2011). We used previously published estimates of the origination times of genes along the *Drosophila* phylogeny (Zhang, Vibranovski, et al. 2010).

All microarray probe sets with multiple matches to the *Drosophila* or *Anopheles* genome were excluded from the analysis. For genes with multiple probe sets, the probe set showing the strongest signal intensity across all samples was selected and all others were excluded. Signal intensities at probe sets with absent calls were arbitrarily set to 1. Array intensity values were $\log_2$ transformed and mean expression values were calculated from three or four replicate arrays for each sample. All of the microarray expression distributions were bimodal, with a lower mode that presumably corresponds to background microarray hybridization to probes matching lowly or nonexpressed genes. Microarray data sets were therefore truncated and expression values in the lower mode were excluded from all analyses except the calculation of τ. For each sample, we normalized expression distributions by the median expression level prior to identifying biased gene sets.

To ascertain the contribution of X-autosome differences in gene expression to the observed deficit of X-linked male-biased genes, we first identified genes expressed predominantly in specific tissues using a method that is less sensitive to gene expression levels than a single direct comparison between two samples (e.g., testes vs. ovaries; Parisi et al. 2003; Sturgill et al. 2007). In particular, we utilized microarray data from 20 different tissues together in a single analysis to identify broadly expressed genes versus those expressed in specific cells and tissues. To measure the degree of tissue specificity, we calculated the metric τ (Yanai et al. 2005) for each gene

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

where E_i is $\log_2$ expression in sample i and max E_i is the maximal $\log_2$ expression level for that gene across all samples.

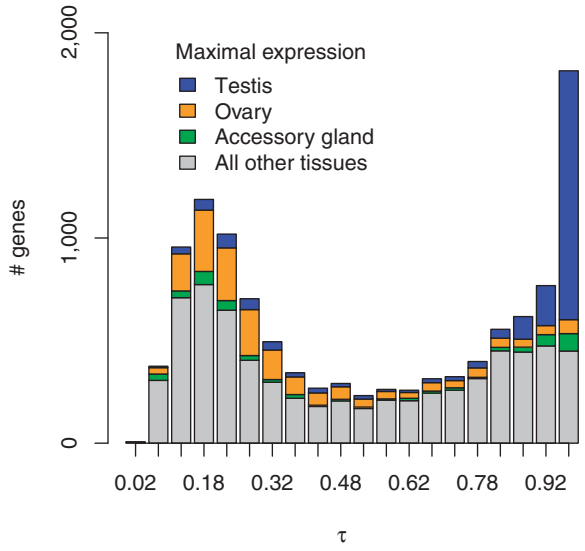

Fig. 1.—The distribution of τ values, a metric of tissue-specific expression, is distinctly bimodal for 11,186 genes measured across 20 larval and adult structures, organs, and tissues. The lower mode corresponds to broadly expressed genes, and the upper mode to highly biased or tissue-specific genes. The distributions of τ values for genes with maximal expression in testis, ovary, or accessory gland are shown in color. The testis is unusual in the number of genes that are expressed exclusively in these cells, whereas ovaries transcribe broadly expressed genes at high levels.

Smoothed distributions in figure 2 were obtained using kernel density estimation and a bandwidth of one. Percent deviations (see tables 1, 3–6) were calculated as $(O - E)/E \times 100$, where O is the observed number of X-linked or autosomal genes, and E is the expected number based on all genes in the genome. All analyses were performed in R (R Development Core Team 2011). Genes on the heterochromatic 4th chromosome were excluded from all analyses.

Results

Genes Expressed Primarily in Testes Are Not Underrepresented on the X Chromosome

The distribution of τ—a measure of tissue specificity (Yanai et al. 2005)—in the FlyAtlas microarray data is bimodal (fig. 1): many genes are either broadly expressed or tissue-specific. Compared with other tissues, the testis is exceptional in the extent to which gene expression in these cells is tissue specific: 67% of all genes with $\tau > 0.95$ are strongly testis biased or testis specific, and 15% of all genes expressed in testis have τ values >0.95 (supplementary table S1, Supplementary Material online; see also Fuller 1998; Chintapalli et al. 2007; Mikhaylova and Nurminsky 2011). In contrast, ~0.5% of genes expressed in other tissues, on average, show $\tau > 0.95$. Tissue specificity is not, however, a general property of germline expression, as ovaries show no enrichment for tissue-specific genes (fig. 1 and supplementary table S1, Supplementary Material online). Instead, gene expression in ovaries is largely characterized by upregulation of broadly expressed genes (Meisel 2011).

We compared the chromosomal locations of tissue-specific genes with that expected by chance. Across three arbitrary values of τ chosen as cutoffs to define tissue specificity, testis-specific genes show no significant departure from expected proportions on the X chromosome versus the autosomes (independently observed by Meisel et al. 2012). In contrast, ovary-specific genes are significantly overrepresented on the X at all three τ cutoffs after correcting for multiple tests, whereas accessory gland-specific genes are significantly underrepresented on the X (table 1 and supplementary table S2, Supplementary Material online). Ovary-specific genes show the largest deviation from random expectation, an enrichment of 105% on the X chromosome at $\tau > 0.9$. We conclude that, using τ as an indicator of tissue-specific expression, there is no evidence for a deficit of X-linked testis-specific genes in *D. melanogaster*. In the germline, the *Drosophila* X chromosome is, if anything, feminized (Parisi et al. 2003; Meisel et al. 2012).

Young genes—those acquired recently by retroduplication, DNA-based duplication or de novo origination—are distributed throughout the genome differently than long-established old genes (Zhang, Vibranovski, et al. 2010). In particular, old male-biased genes are underrepresented on the X chromosome, whereas young male-biased genes are overrepresented on the X chromosome (Zhang, Vibranovski, et al. 2010). We examined the relationship between gene age and expression specific to the male germline. Young genes (defined as <63 Myr old, following Zhang, Vibranovski, et al. [2010]) are both significantly more tissue specific (supplementary fig. 1, Supplementary Material online) and more likely to be testis-specific in expression than old genes (>63 Myr old): 10% of old genes and 39% of young genes are testis specific at a τ cutoff of 0.9 (supplementary table S3, Supplementary Material online). We find that, as with male-biased genes (Zhang, Vibranovski, et al. 2010), young testis-specific genes are significantly overrepresented on the X, whereas old testis-specific genes are significantly underrepresented on the X (supplementary table S4, Supplementary Material online). Together, these complementary deviations balance, such that overall, the number of X-linked testis-specific genes is not different from that expected by chance (table 1).

No Sex Chromosome Dosage Compensation in the *Drosophila* Testis

There are conflicting reports in the literature concerning the status of X chromosome dosage compensation in the *Drosophila* male germline. The first claims of X chromosome demasculinization inferred that average expression levels from the X and autosomes are equal in male and female somatic

Table 1
Chromosomal Location of Tissue-Specific Genes ($\tau > 0.9$)

Tissue	X Chromosome		Autosomes		P (χ^2 Test)
	Observed	% Deviation	Observed	% Deviation	
All genes	**1,793**		**9,321**		
Adult brain	24	27.1	93	−5.2	0.1977
Accessory gland	**5**	**−77.9**	**135**	**15.0**	**0.0001**
Adult crop	2	−50.4	23	9.7	0.2689
Adult eye	11	−28.2	84	5.4	0.2276
Adult fatbody	1	−22.5	7	4.3	0.7800
Adult hindgut	3	−31.1	24	6.0	0.4781
Adult heart	4	−14.5	25	2.8	0.7319
Adult midgut	9	−36.6	79	7.0	0.1320
Adult salivary gland	0	−100.0	13	19.2	0.1138
Adult thoracicoabdominal ganglion	4	3.3	20	−0.6	0.9433
Ejaculatory duct	5	0.0	26	0.0	0.9995
Female (virgin) spermatheceae	2	−38.0	18	7.3	0.4559
Larval CNS	16	60.0	46	−11.5	0.0384
Larval hindgut	4	−51.4	47	9.9	0.1075
Larval midgut	13	−26.7	97	5.1	0.2186
Larval salivary gland	13	34.3	47	−6.6	0.2439
Larval trachea	11	−31.8	89	6.1	0.1629
Larval malpighian tubules	5	−49.2	56	9.5	0.0920
Ovary	**37**	**104.8**	**75**	**−20.2**	**<0.0001**
Testis	221	−2.7	1187	0.5	0.6559

NOTE.—Values in boldface indicate significant P values at FDR = 0.05.

and germline tissues (using whole testis dissections; Parisi et al. 2003; Gupta et al. 2006; Sturgill et al. 2007). It has become clear, however, that X-linked genes have significantly lower expression, on average, than autosomal genes in the *Drosophila* testis (Meiklejohn et al. 2011). Four independent data sets, using two different microarray platforms as well as RNA-seq, and assaying gene expression from whole testes and subtestis dissections, all show that median gene expression values differ significantly between X-linked and autosomal genes (Mann–Whitney $P_{MW} < 0.001$), with X-linked genes showing 1.43- to 1.51-fold (0.52–0.59 on a $\log_2$ scale) lower median expression (table 2). This X-autosome difference is not attributable to the unusual, highly tissue-specific gene expression profile of testis, as the same ~1.5-fold X-autosome difference in expression holds for broadly expressed genes (i.e., those with low τ; table 2). A similar magnitude of differential expression between *X*-linked and autosomal genes is seen in *Drosophila* male-like somatic cells in culture when the DCC is knocked down by RNAi (Hamada et al. 2005; Zhang, Malone, et al. 2010), in early embryos before the DCC is active (Lott et al. 2011), and between aneuploid autosomal genes that differ by 2-fold in copy number (Stenberg et al. 2009; Zhang, Malone, et al. 2010), consistent with an absence of X chromosome dosage compensation in the *Drosophila* male germline (Meiklejohn et al. 2011).

Expression assays using testes from spermatogenesis mutants has, however, raised the possibility that some form of X chromosome dosage compensation may exist in the small population of undifferentiated mitotic spermatogonial cells at the tip of testis (Meiklejohn et al. 2011; Deng et al. 2011). In *bam*-mutant testes, spermatogonia proliferate but fail to differentiate into primary spermatocytes (McKearin and Spradling 1990). An initial analysis of *bam* mutant testes revealed that, instead of a ~1.5-fold X-autosome difference in gene expression, only a ~1.13-fold difference exists (Meiklejohn et al. 2011), leading one study to postulate some form of X chromosome dosage compensation in *Drosophila* spermatogonia (Deng et al. 2011). However, data from an independent study of testis gene expression, using a different *bam* mutant genotype (Chen et al. 2011), reveals the expected 1.5-fold difference (supplementary table S5, Supplementary Material online). These findings have two important implications. First, X-autosome expression differences in *bam* mutant testes clearly depend on the particular *bam* alleles used. Second, while we cannot say definitively which *bam* genotype most accurately reflects expression in wild-type spermatogonia (and which is aberrant), it seems more parsimonious to infer that a 1.5-fold difference is established in spermatogonia and then subsequently maintained throughout the male germline. We therefore conclude that

Table 2

Gene Expression Is Buffered with Respect to Gene Copy Number in *Drosophila*, and X: A Expression Ratios Are Consistent with Haploid Expression in the Male Germline

Cell Type	Log_2 Ploidy Effect[a]	Data Source
Wild-type male germline cells	**A–X**	
Whole testes	0.52	Gan et al. (2010)
Whole testes	0.59	Chintapalli et al. (2007)
Whole testes	0.45	Chen et al. (2011)
Testis apical tips	0.56	Meiklejohn et al. (2011)
Broadly expressed genes		
Whole testes; $\tau < 0.50$	0.67	Chintapalli et al. (2007)
Whole testes; $\tau < 0.40$	0.77	Chintapalli et al. (2007)
Whole testes; $\tau < 0.20$	0.64	Chintapalli et al. (2007)
Cells lacking dosage compensation		
S2 cells (*msl2* RNAi)	0.51	Hamada et al. (2005)
S2 cells (*msl2* or *mof* RNAi)	0.43	Zhang, Malone, et al. (2010)
early embryos	0.54	Lott et al. (2011)
Aneuploid cells	**2-fold copy**	
Deficiency heterozygotes[b]	0.64	Stenberg et al. (2009)
Chromosome 4 monosomy[b]	0.52	Stenberg et al. (2009)
S2-cell aneuploidies	0.58	Zhang, Malone, et al. (2010)

[a]Median A–X expression; all values are significantly different from 0 ($P < 0.001$) by Mann–Whitney test.
[b]RNA extracted from whole adult females.

X chromosome dosage compensation is absent from the vast majority of cells in the testis and, most likely, absent from spermatogonia as well. Further studies using *bona fide* amorphic alleles of *bam* or, alternatively, expression assays on perfectly isolated spermatogonial cells, will be required to definitively assay X-linked and autosomal expression in spermatogonia and male germline stem cells in *Drosophila*. Importantly, whether sex chromosome dosage compensation occurs in the small population of spermatogonial cells has little bearing on the analyses that follow, as it remains clear that most cells in the testes show a ~1.5-fold X-autosome difference in expression.

Global Expression Differences between the X and Autosomes in the Testes Explain the Deficit of Testes-Biased Genes on the X

Consistent with previous reports (Parisi et al. 2003, 2004; Ranz et al. 2003; Sturgill et al. 2007; Gan et al. 2010), direct pairwise comparison of gene expression levels between testis and all other FlyAtlas samples shows a pattern consistent with X chromosome demasculinization—a paucity of X-linked genes overexpressed ≥2-fold in testes—that is highly significant ($\chi^2 \geq 11.8$, $P < 0.001$) and consistent in magnitude (22–40%; tables 3–5; see supplementary table S6, Supplementary Material online; for results with 4- and 8-fold testis-biased genes). The fact that X chromosome demasculinization is seen when using a 2-fold cutoff to identify testis-biased genes but not when using τ raises the possibility that this observation reflects differential gene expression rather than differential gene content. To test this possibility, we transformed X-linked log_2 expression values for each FlyAtlas tissue by the difference between median X and autosomal expression in that tissue (supplementary table S7, Supplementary Material online), equalizing global X and autosomal expression. Following this transformation, testis-biased genes are no longer underrepresented on the X chromosome (table 6 and supplementary table S8, Supplementary Material online). X chromosome demasculinization in the *Drosophila* testis thus appears to depend on the small overall difference in median X versus autosome expression level in the testis.

We performed a second test of the hypothesis that global differences in X versus autosomal expression, specifically a lack of germline X chromosome dosage compensation in testes, can account for apparent X chromosome demasculinization. Experimental impairment of the DCC by RNAi against *msl2* in male-like S2 cell culture combined with whole-genome expression profiling shows that loss of DCC-mediated dosage compensation results in a global decrease in X chromosome expression (Hamada et al. 2005; Zhang, Malone, et al. 2010). We transformed testis gene expression values by the change in expression measured in S2 cells upon RNAi against *msl2* (Hamada et al. 2005). Following this transformation, genes with a 2-fold or greater testis bias are significantly underrepresented ($\chi^2 \geq 7.56$, $P < 0.01$) on the X in 7 of 20 comparisons versus FlyAtlas tissues (supplementary table S9, Supplementary Material online), and the magnitude of the underrepresentation versus these seven tissues ranges from 18% to 22%. This transformation thus reduces but does not wholly eliminate the X chromosome demasculinization.

Table 3

Chromosomal Distribution of Genes with >2-Fold Bias in Testis

Comparison Tissue	X Chromosome		X vs. A
	Observed	% Deviation	*P* (χ^2 Test)
Accessory gland	128	−31.1	<0.0001
Adult brain	144	−31.7	<0.0001
Adult crop	125	−30.9	<0.0001
Adult eye	126	−30.9	<0.0001
Adult fatbody	141	−25.9	0.0001
Adult heart	142	−24.9	0.0002
Adult hindgut	120	−34.6	<0.0001
Adult midgut	138	−28.3	<0.0001
Adult salivary gland	137	−27.9	<0.0001
Adult thoracicoabdominal ganglion	131	−32.9	<0.0001
Ejaculatory duct	129	−24.8	0.0004
Female (virgin) spermatheceae	153	−22.7	0.0005
Larval CNS	126	−33.7	<0.0001
Larval hindgut	124	−29.5	<0.0001
Larval malpighian tubules	152	−22.5	0.0006
Larval midgut	116	−40.6	<0.0001
Larval salivary gland	132	−24.7	0.0004
Larval trachea	125	−32.9	<0.0001
Ovary	163	−22.7	0.0003
Testis	—	—	—

NOTE.—All *P* values are significant at FDR = 0.05.

Table 4

Chromosomal Distribution of Genes with >2-Fold Bias in Ovary

Comparison Tissue	X Chromosome		X vs. A
	Observed	% Deviation	*P* (χ^2 Test)
Accessory gland	186	6.6	0.3443
Adult brain	**215**	**19.8**	**0.0037**
Adult crop	**196**	**16.4**	**0.0203**
Adult eye	**219**	**15.5**	**0.0196**
Adult fatbody	217	20.4	**0.0028**
Adult heart	**240**	**18.9**	**0.0033**
Adult hindgut	209	13.8	0.0406
Adult midgut	217	5.8	0.3621
Adult salivary gland	221	16.6	**0.0126**
Adult thoracicoabdominal ganglion	**226**	**25.6**	**0.0002**
Ejaculatory duct	**230**	**19.9**	**0.0026**
Female (virgin) spermatheceae	230	22.5	**0.0008**
Larval CNS	**114**	**24.4**	**0.0107**
Larval hindgut	177	8.7	0.2236
Larval malpighian tubules	218	8.1	0.2090
Larval midgut	216	3.9	0.5422
Larval salivary gland	180	12.7	0.0797
Larval trachea	162	13.9	0.0712
Ovary	—	—	—
Testis	**392**	**50.1**	<**0.0001**

NOTE.—Values in boldface indicate significant *P* values at FDR = 0.05.

Table 5

Chromosomal Distribution of Genes with >2-Fold Bias in Accessory Gland

Comparison Tissue	X Chromosome		X vs. A
	Observed	% Deviation	*P* (χ^2 Test)
Accessory gland	—	—	—
Adult brain	220	3.9	0.5396
Adult crop	139	7.6	0.3477
Adult eye	173	11.9	0.1052
Adult fatbody	154	7.6	0.3197
Adult heart	180	14.3	0.0498
Adult hindgut	146	9.8	0.2159
Adult midgut	184	11.3	0.1135
Adult salivary gland	144	6.1	0.4352
Adult thoracicoabdominal ganglion	198	4.8	0.4704
Ejaculatory duct	137	9.2	0.2629
Female (virgin) spermatheceae	183	17.9	0.0148
Larval CNS	171	−2.5	0.7190
Larval hindgut	120	6.4	0.4571
Larval malpighian tubules	185	9.3	0.1857
Larval midgut	169	−4.6	0.5043
Larval salivary gland	102	0.8	0.9267
Larval trachea	122	3.7	0.6583
Ovary	245	6.8	0.2610
Testis	**416**	**30.6**	<**0.0001**

NOTE.—Values in boldface indicate significant *P* values at FDR = 0.05.

Table 6

Testis-Biased Genes Are Not Underrepresented on the X Chromosome after Transformation by Differences in Median X versus A Expression Levels

Comparison Tissue	X Chromosome		X vs. A
	Observed (*N* = 1,793)	% Deviation	*P* (χ^2 Test)
Accessory gland	188	1.2	0.5565
Adult brain	212	0.5	0.4642
Adult crop	184	1.7	0.6055
Adult eye	173	−5.3	0.1731
Adult fatbody	174	−8.7	0.0882
Adult heart	185	−2.2	0.3836
Adult hindgut	173	−5.8	0.1316
Adult midgut	198	2.7	0.7318
Adult salivary gland	189	−0.6	0.4572
Adult thoracicoabdominal ganglion	196	0.3	0.4533
Ejaculatory duct	185	7.7	0.7361
Female (virgin) spermatheceae	193	−2.6	0.3773
Larval CNS	195	2.5	0.6258
Larval hindgut	185	5.0	0.9355
Larval malpighian tubules	211	7.5	0.6954
Larval midgut	177	−9.5	0.0306
Larval salivary gland	181	3.1	0.8447
Larval trachea	194	4.0	0.7782
Ovary	253	19.9	0.0458

NOTE.—No *P* values are significant at FDR = 0.05.

Fig. 2.—A demasculinized chromosome was simulated by randomly resampling autosomal $\log_2$ gene expression values with an arbitrary fraction of testis-biased genes removed. 1,000 replicate distributions were generated by resampling autosomal genes with >2-fold greater expression in testes versus ovaries. For each distribution, we simulated a demasculinized X chromosome by sampling 1,793 genes (the number of X-linked genes in these data) with 20%, 40%, or 60% fewer testis-biased genes than would be expected given the proportion of testis-biased genes observed on the autosomes. The resampled distributions (dashed gray lines) are plotted alongside the distributions of expression profiles for all X-linked (orange) and autosomal (blue) genes. Following resampling, the median expression of the simulated demasculinized X is significantly greater ($P < 0.05$) than the median observed expression for the true X in all 1,000 replicates for all three levels of demasculinization.

These results suggest either that the lack of sex chromosome dosage compensation explains a large fraction, but not all, of the demasculinized X; or alternatively, that RNAi knock-down of *msl2* in these experiments may not completely abolish MSL-dependent dosage compensation (Hamada et al. 2005).

Simulating a Demasculinized X Chromosome Does Not Recapitulate X Autosome Differences in Expression in the Testes

So far, these analyses show that the lack of sex chromosome dosage compensation in the male germline can account for most, if not all, of the underrepresentation of testis-biased genes on the X chromosome. We next tested the inverse hypothesis: can the difference in median expression between the X and the autosomes be explained by an evolved difference in the density of testis-biased or testes-specific genes? We tested this possibility by simulating a demasculinized X chromosome from the FlyAtlas testis microarray data. We randomly sampled autosomal genes, filtered an arbitrary fraction (20%, 40%, or 60%) of testis-biased genes, and then compared the distributions of $\log_2$ expression values from the simulated demasculinized chromosome with that observed for the X chromosome. The range of demasculinization simulated (20–60%) encompasses previously inferred degrees of demasculinization (Parisi et al. 2003; Ranz et al. 2003; Sturgill et al. 2007), as well as those estimated here (table 3). We find that after removing 20%, 40%, or 60% of testis-biased genes (≥2-fold overexpressed in testes relative to ovaries), the distribution of expression levels for the remaining genes shifts (fig. 2), but the median remains significantly greater ($P_{MW} < 0.05$) than that for X-linked genes in 1,000/1,000 resampled distributions for each reduction in testis-biased gene content (20%, 40%, or 60%). Similar results hold for genes with a ≥4-fold or ≥8-fold testis bias (data not shown). These analyses show that this method of demasculinizing the autosomes does not produce a gene expression profile like that of the X chromosome in the testes.

Drosophila Accessory Gland and Anopheles Testis Show X Chromosome Demasculinization

Somatic tissues in *Drosophila* have been reported to show a deficit of X-linked male-biased genes (Parisi et al. 2003; Sturgill et al. 2007; Bachtrog et al. 2010), and accessory gland proteins in particular are underrepresented on the X chromosome (table 1; Wolfner et al. 1997; Swanson et al. 2001). However, unlike the testis, the accessory gland shows a clear deficit of X-linked genes that cannot be explained by globally reduced expression of the X (tables 1 and 5, supplementary table S7, Supplementary Material online). This observation indicates that both X chromosome regulation and gene content differ between male-specific germline and somatic cells in *Drosophila*, as in the testis the X chromosome is neither dosage compensated nor demasculinized for gene content, whereas in the accessory gland the X is both dosage compensated and demasculinized.

As a complementary comparison, we performed a phylogenetically independent test of X chromosome demasculinization in the mosquito, *A. gambiae*, a species that diverged from *D. melanogaster* >250 Ma (Gaunt and Miles 2002) and that has an independently evolved heteromorphic XY sex chromosome system (Toups and Hahn 2010). By analyzing recently published gene expression data from adult *A. gambiae* tissues, including testis, ovaries, and male accessory glands (Baker et al. 2011), we confirm that—in contrast to *Drosophila*—a strong and significant underrepresentation (76–88% below expectation, $P < 0.0001$) of testis-specific genes exists

on the *Anopheles* X chromosome (Baker et al. 2011). This pattern is restricted to testis-specific genes, as the *Anopheles X* shows a significant overrepresentation of tissue-specific genes expressed in accessory gland, male and female heads, female salivary gland, and female malpighian tubules, suggesting that tissue-specific genes may be generally enriched on the *Anopheles* X chromosome (supplementary table S10, Supplementary Material online).

In *Anopheles* testes, median X chromosome expression is 1.78-fold lower than median autosomal expression ($P_{MW} < 0.0001$; supplementary table S11, Supplementary Material online), suggesting that, as in *Drosophila*, the X chromosome is not dosage compensated in the male germline (Baker and Russell 2011). As in *Drosophila*, we observe a significant deficit of X-linked genes with ≥2-fold testis-bias in *Anopheles* (supplementary table S12, Supplementary Material online). However, unlike *Drosophila,* we observe a significant deficit of testis-biased genes on the *Anopheles* X even after normalization for median X versus autosomal expression levels (supplementary table S13, Supplementary Material online). *Anopheles* X chromosome demasculinization therefore does not depend on differences in X versus autosomal expression in the male germline. These analyses from other tissues and species show that demasculinization can occur when the X and autosomes have equal expression levels (e.g., *Drosophila* accessory gland) or disparate expression levels (e.g., *Anopheles* testis).

Discussion

Three kinds of analysis fail to support the notion that gene content on the *Drosophila* X chromosome is demasculinized in the testis. First, when testis-specific genes are identified in *D. melanogaster* via microarray analysis of 20 different larval and adult structures (Chintapalli et al. 2007), as opposed to via pairwise comparison of testes versus ovaries or whole males versus whole females, there is no evidence for a demasculinized X chromosome (table 1; Meisel et al. 2012). Second, normalizing for the global difference in X-autosome expression in the testis largely eliminates its seemingly demasculinized expression profile (table 6 and supplementary table S8, Supplementary Material online). Third, simulating demasculinization on the autosomes by simply removing testis-biased genes fails to produce a gene expression profile similar to that observed for the X chromosome in the testis (fig. 2). We therefore conclude that the apparent demasculinization of the *Drosophila* X chromosome—at least for genes expressed in testis, which account for the vast majority of sex-biased genes assayed in whole flies (Parisi et al. 2003, 2004; Ranz et al. 2003; Sturgill et al. 2007)—is largely explained by the overall reduced expression from the X chromosome.

We further conclude that the globally reduced expression from the X relative to the autosomes in the testis is most simply explained by an absence of sex chromosome dosage compensation in the male germline. If this conclusion is correct, once the simple ploidy difference between the X and the autosomes is accounted for, the statistical underrepresentation of testis-biased genes on the X chromosome disappears. In contrast, other male-specific tissues appear to show robust evidence for demasculinized gene content regardless of whether they are dosage compensated (*Drosophila* accessory gland and *Anopheles* testis, respectively). The *Anopheles*–*Drosophila* comparison shows, further, that the genomic distributions of genes with sex- and tissue-specific expression can evolve to be lineage specific.

These results bear on our understanding of sex chromosome evolution and gene expression in *Drosophila*. Sex chromosome dosage compensation in *Drosophila* involves large-scale chromatin remodeling of the X chromosome (Gelbart and Kuroda 2009), and this has been hypothesized to impose constraints on the evolution of X-linked gene expression (Vicoso and Charlesworth 2009; Bachtrog et al. 2010; Mikhaylova and Nurminsky 2011). Our results suggest a reinterpretation of some of these consequences of sex chromosome dosage compensation. First, a recent study (Mikhaylova and Nurminsky 2011) suggested that all tissue-specific genes—not just testis-specific ones—are underrepresented on the X chromosome because sex chromosome dosage compensation interferes with tissue-specific regulation of X-linked genes. Using τ cutoffs of 0.85, 0.90, and 0.95, we find that only the accessory gland shows a significant deficit of tissue-specific genes on the X (table 1 and supplementary table S2, Supplementary Material online). Pooling across all tissues, we find no significant difference in the proportion of tissue-specific genes on the X and the autosomes ($\chi^2 \leq 1.99$, $P > 0.158$), although we do find a significant excess of broadly expressed X-linked genes ($\tau < 0.4$, $\chi^2 = 5.26$, $P = 0.022$), which is consistent with the hypothesis that dosage compensation interferes with repression of tissue-specific genes in the wrong cell types (Mikhaylova and Nurminsky 2011).

Second, two comparisons of previously published data on sex-biased gene expression and DCC binding in cell culture concluded that dosage compensation may specifically limit the evolution of male-biased gene expression (Vicoso and Charlesworth 2009; Bachtrog et al. 2010). Both studies relied on previous reports that sex chromosome dosage compensation exists in the testes (Parisi et al. 2003; Gupta et al. 2006; Sturgill et al. 2007). Vicoso and Charlesworth (2009) found that male-biased genes with higher absolute expression are more strongly depleted from the X than lowly expressed male-biased genes. We suggest that the negative relationship between absolute expression of male-biased genes and X-linkage is more simply explained by the absence of dosage compensation in the testes. Bachtrog et al. (2010) found that both germline and somatic male-biased genes are located significantly farther from sequence motifs that recruit the DCC to the X chromosome and less likely to be

bound by the DCC than either unbiased or female-biased genes. The underlying causes of this pattern may differ between the testes and the soma. In the testes, there may be no selection to maintain DCC recruitment motifs near genes expressed primarily in spermatocytes, as DCC-mediated dosage compensation is absent in the male germline (Rastelli and Kuroda 1998). In the soma, if this pattern is largely due to the accessory gland—which shows both X chromosome dosage compensation and demasculinization—then constraint resulting from DCC function would seem a viable hypothesis. However, it is also possible that both germline and somatic male-biased genes are not bound by the DCC in cell culture simply because they are not expressed in the particular cells in culture, as the DCC, once it has localized to the X chromosome, largely binds to expressed genes (Alekseyenko et al. 2006).

Finally, two other observations that raise doubts about the rationale for the evolutionary demasculinization of the X chromosome are made more explicable in light of the present results. One is that a curious and unexplained discrepancy has existed between the distributions of genes with testis-biased or testis-specific expression, which supposedly avoid the X, and genes that are essential for male fertility, which are uniformly distributed throughout the genome and do not avoid the X (Lindsley and Lifschytz 1972). The present results suggest that there is no discrepancy—testis-biased, testis-specific, and male-fertility essential genes are all uniformly distributed. The other is that multiple patterns of gene movement and origination are difficult to reconcile with X chromosome demasculinization. Recent studies of interchromosomal retroduplication in *Drosophila* have confirmed the previously established X→autosome formation bias but show that when the parent copies are lost, both parent genes and retroduplicates tend to be female biased (Metta and Schlotterer 2010). When parent copies are retained, retroduplicates tend to be testis biased regardless of the direction of movement (i.e., autosome→X and autosome→autosome; (Meisel et al. 2009). Thus, sexual antagonism may not be necessary to explain the X→autosome bias in retrogene formation, and it is unclear what role, if any, biased gene movement has in shaping X chromosome gene content. The lack of detectable MSCI in *Drosophila* (Meiklejohn et al. 2011; Mikhaylova and Nurminsky 2011) indicates that it is unlikely to provide the selective force behind biased gene movement in this genus. Furthermore, the excess of X linkage and testis expression observed among young, recently evolved genes (Zhang, Vibranovski, et al. 2010; supplementary tables S3 and S4, Supplementary Material online), particularly those that form de novo from previously noncoding sequences (Levine et al. 2006), seems difficult to reconcile with the notion that the X chromosome is an unfavorable location for genes that function primarily in the male germline.

Taken together, our results imply that, at least in *Drosophila*, models based on MSCI and sexual antagonism are not necessary to explain the X-autosome difference in the density of genes with testis-biased expression. Indeed, the best evidence for the sex-specific adaptation of the sex chromosomes in *Drosophila* comes from the concentration of male fertility-essential genes on the Y chromosome (Brosseau 1960; Kennison 1981), which resides in males exclusively, and from the enrichment of ovary-specific genes on the X chromosome (table 1 and supplementary table S2, Supplementary Material online; see also Parisi et al. 2003; Sturgill et al. 2007; Meisel et al. 2012), which resides in females two-thirds of the time.

Supplementary Material

Supplementary figure S1 and tables S1–S13 are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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