Sex-Specific Effects of *Cis*-Regulatory Variants in *Drosophila melanogaster*

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ABSTRACT Sexual dimorphism at the level of gene expression is common and well documented, but much less is known about how different *cis*-regulatory alleles interact with the different *trans*-regulatory environments present in males and females. Here we show that sex-specific effects of *cis*-regulatory variants are common in *Drosophila*.

A hallmark of dioecious organisms is sexual dimorphism, phenotypic differences between males and females of a species such as size, coloration, and behavior. Differences in these organism-level exophenotypes are governed by sexual dimorphism in underlying endophenotypes including the regulation of gene expression (reviewed in Williams and Carroll 2009). Gene regulation is central to sexual dimorphism because males and females carry the same genome, except for their sex chromosomes. Indeed, the extent to which the genome is differently expressed in the two sexes is quite striking—estimates in *Drosophila* suggest that approximately half of the genes in the genome are expressed differently in males and females (Jin *et al.* 2001; Gnad and Parsch 2006; Innocenti and Morrow 2010).

Mechanistically, the regulation of gene expression is governed by the interaction of *cis*-regulatory DNA sequences at each gene with *trans*-regulatory proteins and RNAs present in each cell (reviewed in Wray *et al.* 2003); the same *cis*-acting sequences have different activities in the different *trans*-regulatory environments of males and females. But, do sex-specific differences in the *trans*-regulatory environment generally have similar effects on alternative *cis*-regulatory alleles of a gene? Or, put another way, how often do *cis*-regulatory variants have sex-specific effects? A recent QTL study of expression variation in *D. melanogaster* found that sex-specific *trans*-regulatory factors appear to often have different effects on alternative *cis*-regulatory alleles (Massouras *et al.* 2012).

Here, we investigate the magnitude of such *cis*-by-sex effects and compare them to the frequency and magnitude of *cis*-by-trans effects from other sources. To do this, we used pyrosequencing (Ahmadian *et al.* 2000) to measure relative allele-specific expression for 11 randomly selected autosomal genes in male and female F_1 progeny from reciprocal crosses between the highly inbred *Drosophila melanogaster* lines *zhr* and *z30* (Begun and Aquadro 1993; Sawamura *et al.* 1993; Wu *et al.* 1995; Ferree and Barbash 2009; Coolon *et al.* 2012). Relative allele-specific expression in heterozygous genotypes provides a direct readout of relative *cis*-regulatory activity (Cowles *et al.* 2002; Wittkopp *et al.* 2004). These reciprocal crosses produced four genetically distinct progeny with identical autosomal genotypes (*i.e.*, heterozygous for the *zhr* and *z30* alleles at all autosomal loci) that differ in the identity of their sex chromosomes and/or the parent of origin for all of their chromosomes (Figure 1A). For each genotype, RNA and genomic DNA were extracted from four biological replicates containing 20 whole flies (7–10 days old) each and analyzed by pyrosequencing using gene-specific primer sets (see supporting information Table S1) and protocols described in Wittkopp (2011).

Pairwise comparisons among these four genotypes resulted in six tests for differences in relative *cis*-regulatory activity between alleles of autosomal genes in different *trans*-regulatory backgrounds (Figure 1, B–F). First, we compared female progeny from reciprocal crosses, which are genetically identical except for any epigenetic marks resulting from the maternal and paternal transmission of alleles

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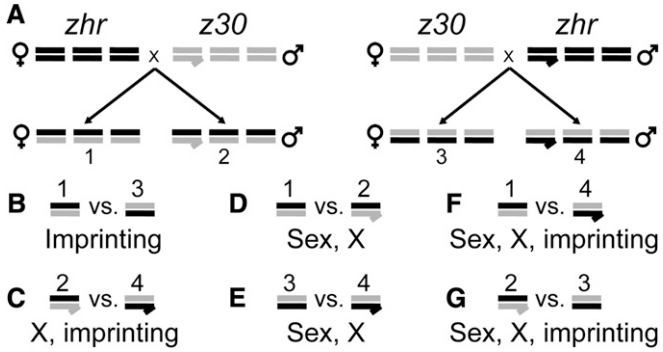

Figure 1 Separating the effects of genomic imprinting, epistatic interactions, and sexual dimorphism using reciprocal crosses. (A) Chromosomes present in the parental strains and F_1 offspring (excluding the "dot" 4th chromosome) are shown with chromosomes derived from *zhr* (solid) and chromosomes derived from *z30* (shaded). Note that all four types of offspring are heterozygous for all autosomes. (B–G) Six comparisons were performed, contrasting each type of offspring with each other type. For each genotypic type, only the sex chromosomes are shown. The source(s) of interactions potentially affecting relative *cis*-regulatory activity of autosomal genes in each comparison is shown. Imprinting, genomic imprinting; X, epistatic interactions with variable X- or Y-linked loci; and sex, sexually dimorphic *trans*-regulatory factors.

known as genomic imprinting (Figure 1B). Next, we compared male progeny from reciprocal crosses, in which relative *cis*-regulatory activity could differ because of genomic imprinting and/or differences in X and Y chromosome genotypes; genetic differences between the *zhr* and *z30* sex chromosomes have the potential to interact epistatically with *cis*-regulatory differences between the *zhr* and *z30* alleles of the autosomal genes tested (Figure 1C). In the third and fourth comparisons, we examined male and female progeny from the same cross (Figure 1, D and E). Differences in relative *cis*-regulatory activity of autosomal genes in these cases could be caused by epistatic effects of *trans*-acting variants located on the X and/or Y chromosomes and/or differences in the *trans*-regulatory environment between males and females resulting from sexual dimorphism (*i.e.*, the same pairs of *cis*-regulatory variants react differently to the *trans*-regulatory environment of males and females resulting in a sex×*cis* interaction). Finally, in the fifth and sixth comparisons, we contrasted male progeny from one cross with female progeny from the reciprocal cross (Figure 1, F and G). Differences in relative *cis*-regulatory activity of autosomal genes in these comparisons could come from genomic imprinting, epistatic effects of genetic differences on the sex chromosomes, and/or sexually dimorphic *trans*-regulation. In all cases, if relative activity of the *zhr* and *z30* *cis*-regulatory alleles for autosomal genes is independent of the difference(s) in *trans*-acting environment, then relative allele-specific expression of these genes should be similar between the two genotypes compared. If, however, the *cis*- and *trans*-regulatory differences interact, relative allele-specific expression should differ between genotypes.

Measures of relative *cis*-regulatory activity (Y_{ijk}) were calculated from the pyrosequencing data as $log_2(zhr/z30)$ for each gene (i) in each sex (j) from each cross (k), as described in Wittkopp (2011). These data were then fitted to the following linear model using proc MIXED in SAS v10.3 (Cary, NC): $Y_{ijk} = \mu + Sex_j\,(Gene_i) + Cross_k\,(Sex_j\,(Gene_i)) + \varepsilon$. This model controlled for the differences in *cis*-regulatory activity among genes and allowed us to focus on the effects of different *trans*-regulatory backgrounds on relative *cis*-regulatory activity of the autosomal *zhr* and *z30* alleles. We examined the effects of genomic imprinting, epistasis with *trans*-acting variants on the sex chromosomes, and sex×*cis* interaction with sexually dimorphic *trans*-regulatory environments on individual genes using the differences in least-squares means and 95% confidence intervals for these differences derived from this model. An interaction was considered statistically significant for a gene if the 95% confidence interval of the difference did not include zero. This is a conservative test for the absence of an interaction because it does not control for the increased false positive rate resulting from multiple testing.

Comparing females from reciprocal crosses (Figure 1B), we found no statistically significant evidence of genomic imprinting for any gene (Figure 2A), consistent with prior studies (Wittkopp *et al.* 2006; Coolon *et al.* 2012). In the comparison where relative *cis*-regulatory activity could be affected by either imprinting or genetic differences between X and/or Y chromosomes (Figure 1C), one gene showed a statistically significant effect (Figure 2B). Given the absence of evidence for imprinting in the first comparison, we conclude that this difference most likely resulted from epistatic effects of one or more *trans*-acting loci that differ between the *zhr* and *z30* alleles of one or both sex chromosomes. Previous studies provide mixed evidence for this type of epistasis: an intraspecific comparison of *D. melanogaster* females found no evidence for it among the eight genes tested (Wittkopp *et al.* 2008), whereas a study of interspecific *Drosophila* hybrids (*D. yakuba* and *D. santomea*) found evidence for it affecting 19 of the 22 genes tested (Llopart 2012). We observed much larger differences in relative *cis*-regulatory activity in all comparisons between males and females (Figure 1, D–G), with significant differences observed for 6 of 11 genes tested in at least one of the four comparisons (Figure 2, C–F). The statistical significance of the difference in relative *cis*-regulatory activity varied among comparisons for some genes, but the relative magnitude of the differences was generally consistent among genes in all four comparisons (Figure 2, C–F). This is consistent with differences in *trans*-regulation between males and females that are similar in all four contrasts and primarily responsible for the differences in relative *cis*-regulatory activity observed. Statistical significance of the $Sex_j\,(Gene_i)$ and $Cross_k\,(Sex_j\,(Gene_i))$ terms in the full model provide further support for these conclusions (Table 1): after controlling for gene-specific effects, differences between sexes (reflecting sexual dimorphism) explained much more of the

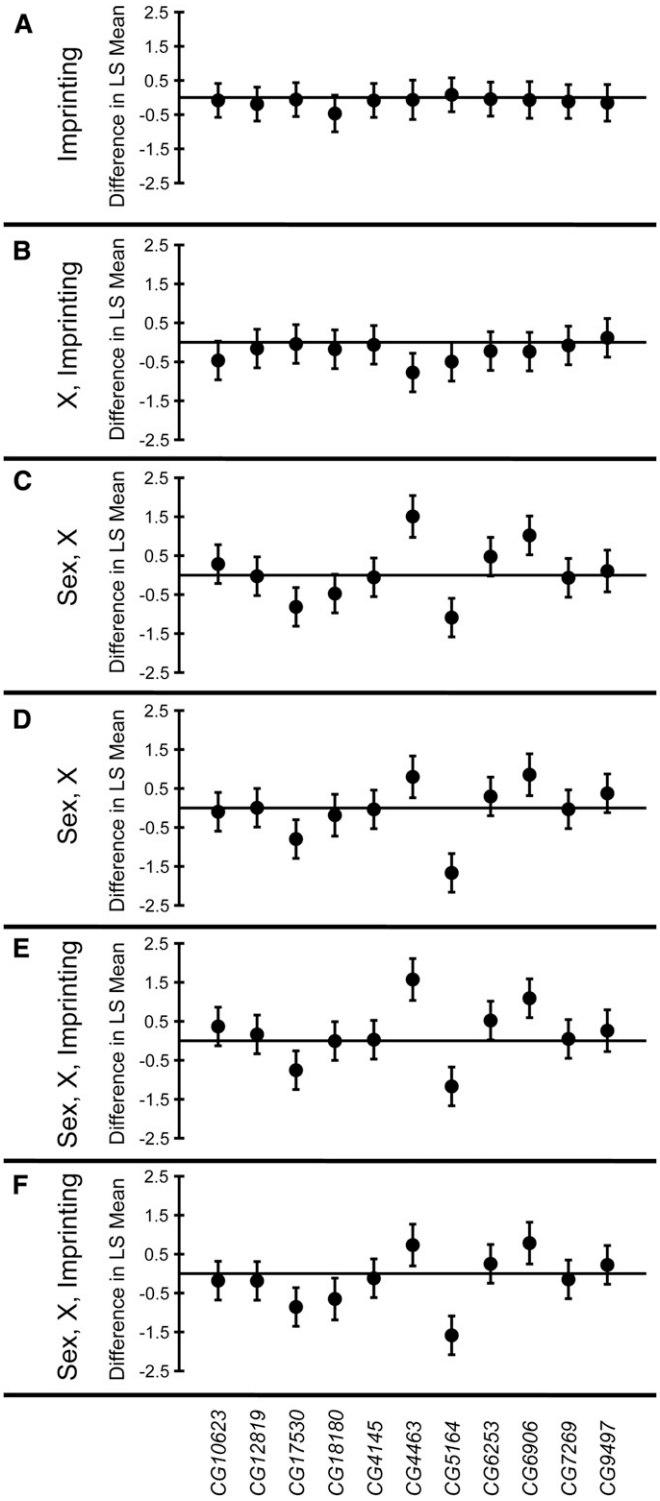

Figure 2 Relative *cis*-regulatory activity differed the most between males and females. For each of the six comparisons described in Figure 1, B–G, the difference in relative *cis*-regulatory activity for each of the 11 genes tested is shown using the least-squares means (LS means) and corresponding 95% confidence intervals derived from the general linear model described in the main text. A–F correspond to B–G in Figure 1, respectively. In each case, the difference was considered to be statistically significant if zero was not contained within the 95% confidence interval. The potential causes of significant differences are indicated for each comparison. Imprinting, genomic imprinting; X, epistatic interactions with variable X- or Y-linked loci; and sex, sexually dimorphic *trans*-regulatory factors.

Table 1 Summary of effects from the general linear model

Effect	d.f.	Sum of squares	Mean square	*F*	*P*-value
Sex(gene)	21	75.82	3.61	119.23	<1E-25
Cross(sex(gene))	22	3.08	0.14	4.63	1.40E-08

d.f.. degrees of freedom.

total variation in relative *cis*-regulatory activity ($F = 119$) than the combined effects of genomic imprinting and epistasis with X- and Y-linked variation captured by the reciprocal crosses ($F = 5$).

Sexual dimorphism creates differences in gene expression between males and females (Jin *et al.* 2001; Gnad and Parsch 2006; Innocenti and Morrow 2010), and the data presented here show that these sex-specific *trans*-regulatory environments often interact differently with alternative *cis*-regulatory alleles of a gene. This suggests that many *cis*-regulatory polymorphisms have different effects in males and females. Interactions between sexually dimorphic *trans*-regulatory environments and species-specific *cis*-regulatory alleles also were recently observed between *D. simulans* and *D. mauritiana* using a different experimental design (Meiklejohn *et al.* 2013), indicating that these effects are not limited to *cis*-regulatory variants segregating within a species. Furthermore, while our observations are based on a small subset of the genome, the genes used are not enriched for particular functional groups, chromosomal location, or magnitude of *cis*-regulatory differences (data not shown), suggesting that the set is unbiased and that sex×*cis*-regulatory variant interactions are common, consistent with Massouras *et al.* (2012). These types of interactions can result, for example, from *cis*-regulatory variants that affect binding sites for *trans*-regulatory factors that differ between the two sexes (Williams and Carroll 2009; Cooley *et al.* 2012), as was reported for the *Drosophila desatF* gene (Shirangi *et al.* 2009).

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Table S1 Pyrosequencing assays for genotyping and quantification of allelic expression ratios. Pyrosequencing assays were developed for 47 genes. For each assay, one forward and one reverse PCR primer were produced and amplicon lengths for PCR reactions are reported. One of the primers in each reaction is biotinylated for capture for pyrosequecing. A third primer for each assay is used in the pyrosequencing reaction. The sequence analyzed with differentiating SNPs indicated by ambiguity codes are shown. For each assay, custom dispensation orders for the pyrosequencing reaction were developed, zhr and z30 alleles are indicated, and the formula used for analysis are listed.

Table S1 is available for download at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.156331/-/DC1.