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# The roles of *cis*- and *trans*-regulation in the evolution of regulatory incompatibilities and sexually dimorphic gene expression

Colin D. Meiklejohn[1*], Joseph D. Coolon[2], Daniel L. Hartl[3] and Patricia J. Wittkopp[2]

[1]Department of Biology, University of Rochester, Rochester NY 14627

[2]Department of Ecology and Evolutionary Biology and Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor MI

[3]Department of Organismic and Evolutionary Biology, Harvard University, Cambridge MA

*Corresponding author
Department of Biology
Indiana University
Jordan Hall
1001 East Third St.
Bloomington, IN 47405
(ph) 401-374-0467 (fax) 812-855-6705
email: cmeiklej@indiana.edu

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**Abstract**

Evolutionary changes in gene expression underlie many aspects of phenotypic diversity within and among species. Understanding the genetic basis for evolved changes in gene expression is therefore an important component of a comprehensive understanding of the genetic basis of phenotypic evolution. Using interspecific introgression hybrids, we examined the genetic basis for divergence in genome-wide patterns of gene expression between *Drosophila simulans* and *D. mauritiana*. We find that *cis*-regulatory and *trans*-regulatory divergence differ significantly in patterns of genetic architecture and evolution. The effects of *cis*-regulatory divergence are approximately additive in heterozygotes, quantitatively different between males and females, and well predicted by expression differences between the two parental species. In contrast, the effects of *trans*-regulatory divergence are associated with largely dominant introgressed alleles, have similar effects in the two sexes, and generate expression levels in hybrids outside the range of expression in both parental species. Although the effects of introgressed *trans*-regulatory alleles are similar in males and females, expression levels of the genes they regulate are sexually dimorphic between the parental *D. simulans* and *D. mauritiana* strains, suggesting that pure-species genotypes carry unlinked modifier alleles that increase sexual dimorphism in expression. Our results suggest that independent effects of *cis*-regulatory substitutions in males and females may favor their role in the evolution of sexually dimorphic phenotypes, and that *trans*-regulatory divergence is an important source of regulatory incompatibilities.

## Introduction

Phenotypic evolution occurs both by changes to RNA and protein sequences and by changes in the level at which these molecules are expressed within cells. Evolution of gene regulation was hypothesized (Britten and Davidson 1969; King and Wilson 1975) and has been demonstrated to constitute an important component of divergence among species and variation within populations, including significant contributions to human disease (Carroll 2005; Wray 2007; Fay and Wittkopp 2008; Romero et al. 2012). Broadly speaking, gene regulation requires the activity of *trans*-acting factors, which directly or indirectly regulate gene expression via RNA or protein intermediates, and *cis*-acting sequences, which modulate localization of *trans*-acting factors to DNA and their effects on the expression of nearby genes. Studies of the genetic control of gene expression indicate that there is abundant variation within natural populations in both *cis* and *trans*-regulation, and that both modes contribute to adaptation and divergence between species (Wray 2007; Fay and Wittkopp 2008). However, *cis*- and *trans*-regulation have been hypothesized to differ in important genetic and evolutionary properties. First, *cis*-regulatory variants have been observed to have quantitatively larger effects on expression than *trans*-regulatory variants (Brem et al. 2002; Hughes et al. 2006; Zhang et al. 2011; Gruber et al. 2012), although there are exceptions (Genissel et al. 2008; Emerson et al. 2010). Second, a greater proportion of *cis*-regulatory variants have additive effects on expression than *trans*-acting variants, which are more likely to be dominant or recessive (Wray 2007; Lemos et al. 2008a; McManus et al. 2010; Zhang et al. 2011; Gruber et al. 2012). Third, *cis*-regulatory mutations have been hypothesized to have fewer pleiotropic effects than mutations in transcriptional regulators, which can affect the expression of hundreds of genes (Stern 2000). Because additive phenotypic effects increase the efficacy of selection and reduced pleiotropy can decrease deleterious side-effects of mutations, it has been proposed that *cis*-regulatory changes will be enriched between species relative to their frequency within species (Lemos et al. 2008a; Wittkopp et al. 2008; Emerson et al. 2010). However, the relative contributions of *cis*- and *trans*-regulation to variation in gene expression within and among species varies extensively between studies (*e.g.* (Li and Burmeister 2005), indicating that estimates of

these quantities are influenced by either the genotypes or methodologies used to measure them.

Gene regulation is central to the evolution and development of sexual dimorphism. As males and females of the same species carry the same genes (except for differentiated sex chromosomes), phenotypic differences between the sexes must result from differential expression of a single genome. Sexual dimorphism is a universal feature of dioecious animals**,** and estimates of the number of genes differentially expressed between adult male and female *D. melanogaster* range from 4,000 (Gnad and Parsch 2006) to more than 17,000 (Innocenti and Morrow 2010); however, allometric differences in tissue size relative to body size between males and females can give the appearance of sex-biased expression in the absence of sexually dimorphic transcription when expression is assayed from whole animals (Ranz et al. 2004). Mutations may have different selective effects in males and females, due to global differences between the sexes in gene regulation and chromatin environments (Liu et al. 2005; Gelbart and Kuroda 2009), sex-specific reproductive strategies (Lawson Handley and Perrin 2007), and the need to express genes in sex-specific cells such as the gonads (Chintapalli et al. 2007). A frequently cited model of the evolution of sexually dimorphic gene expression invokes such "sexually-antagonistic" alleles that are beneficial in one sex but detrimental in the other (Rice 1984). The fixation of a secondary mutation that changes expression of the antagonistic allele in the disfavored sex could simultaneously resolve the fitness conflict and generate sexually dimorphic expression. Whole-genome expression analyses have claimed to identify sexually antagonistic phenotypes in *Drosophila* (Connallon and Knowles 2005; Innocenti and Morrow 2010); however, neither study determined the genetic basis of sexually dimorphic expression, leaving key aspects of this model untested (Fry 2010).

To identify the genetic basis for regulatory divergence between closely related species, we conducted a genome-wide expression analysis in introgression hybrids between two sister species of *Drosophila*. Previous studies of gene expression in interspecific hybrids of *Drosophila* have focused largely on the $F_1$ generation, assayed gene expression in sterile hybrid animals, and been restricted to one sex (Ranz et al. 2004; Wittkopp et al.

2004; Moehring et al. 2007; Wittkopp et al. 2008; Graze et al. 2009; Lu et al. 2010; McManus et al. 2010). In contrast, we focus here on advanced generation hybrids between *D. simulans* and *D. mauritiana*, which produce fertile $F_1$ females, allowing genetic material to be moved between these species. We used microarrays to measure gene expression in genotypes derived from an inbred strain of *D. simulans* except for a small region on the third chromosome that was introgressed from an inbred strain of *D. mauritiana*. Males and females of all genotypes used here are viable and fertile, allowing us to assay expression in (relatively) phenotypically normal adults of both sexes. Our study constitutes a fine-scale genetic analysis of interspecific divergence in genome-wide gene expression and allows us to measure the effects of divergent regulatory loci in both sexes. We find that divergent gene expression caused by the introgressed genomic segments act largely via *trans*-regulation, that *cis*- and *trans*-regulatory divergence differ in multiple aspects of inheritance, and that *cis*-regulatory divergence is enriched for sexually dimorphic effects on expression.

## Results

### *Detecting divergent regulation in introgression genotypes*

We assayed genome-wide gene expression using microarrays in virgin adult male and female flies of seven hybrid genotypes that introduce a combined ~ 9.3 Mb on chromosome arm 3L from *D. mauritiana* into an otherwise *D. simulans* genetic background. Quantitative trait locus (QTL) mapping identified genes that are differentially expressed between introgression genotypes and the parental *D. simulans* strain with expression phenotypes that are statistically significantly linked to a marker within the introgressed region (Figure 1). We refer to genes with genetic evidence for regulatory differences between species as *divergently regulated*. Divergent regulation of genes located outside the introgressed region results from functionally divergent *D. mauritiana* alleles of *trans*-acting factors located within the introgressed region. Divergently regulated genes located within the introgressed region and close to a genetic

marker linked to the regulatory effect are candidates for divergent *cis*-regulatory sequences.

In both sexes, we observe a significant enrichment of divergently regulated genes located within the introgressed region (Table 1) resulting from *cis*-regulatory divergence between these two species. This inference was confirmed by measurements of allele-specific expression via pyrosequencing in introgression heterozygotes generated by crossing introgression genotypes to the parental *D. simulans* strain (see Methods). We measured allele-specific expression of 90 genes in males; 85 of these genes also gave reproducible results in females. Among all genes assayed for allele-specific expression, 76% and 71% showed significant differences in expression between the *D. simulans* and *D. mauritiana* alleles in males and females, respectively (Supplemental Table 1), indicating *cis*-regulatory divergence at these loci. The allele-specific ratios obtained from pyrosequencing and the effects estimated from the microarrays are similar (Supplemental Figure 1; $\rho = 0.744$ and 0.719 in males and females, respectively; $P < 0.0001$). We excluded those genes where pyrosequencing failed to confirm the microarray results from our analyses of *cis*-regulatory divergence.

Differentially expressed genes located within the introgressed region but unlinked from the genetic marker associated with the regulatory effect are regulated in *trans*. In males and females respectively, 40% and 48% of divergently regulated genes located in the introgression are regulated in *trans*; however, many more introgressed genes are presumably divergently regulated by *trans*-factors located elsewhere in the genome. Genome-wide, we detect 665 genes in males and 490 genes in females that are divergently *trans*-regulated (Table 1) as a result of evolutionary divergence in a region comprising less than 10% of the *Drosophila* euchromatic genome.

*Sexual dimorphism in divergent gene regulation*

At a *P*-value of $2e^{-4}$, which corresponds to a false discovery rate (FDR) of 0.02 in males and 0.03 in females (see Methods), we detect 737 divergently regulated genes in males

and 536 in females (Table 1), indicating that a significantly larger fraction of the genome is differentially expressed in males than in females as a result of the introgressed segments ($\chi^2 = 126$, $P < 0.001$). This excess in males is observed among genes regulated both in *trans* and *cis*, is robust to varying the FDR (Supplemental Table 2), and consists mainly of expression effects of small magnitude (Supplemental Figure 2). Previous studies have shown that the expression of genes transcribed mainly or specifically in the testes diverges rapidly between species and is highly variable within species (Meiklejohn et al. 2003; Ranz et al. 2003; Zhang et al. 2007; Brawand et al. 2011). Although 100 genes divergently regulated in males are expressed specifically in testes in *D. melanogaster* (Chintapalli et al. 2007), testis-specific genes are not overrepresented among divergently regulated genes ($\chi^2 = 2.40$, $P > 0.05$, Supplemental Table 3). Additionally, after excluding germline-specific genes there remain significantly more divergently regulated genes in males than in females, indicating that the excess of divergent regulation seen in males cannot be solely attributed to rapid gene expression evolution in the male germline.

A large majority (~80%) of genes detectably expressed in both sexes show significant divergent regulation in either males or females but not both (Supplemental Table 4). However, direct comparison of the homozygous effects of introgressed alleles on gene expression in males and females (Figure 2A), regardless of the statistical significance of QTL linkage, suggests that requiring a significant association in both sexes overestimates the extent of genetic independence in divergent regulation between the sexes. In general, the largest expression effects are similar in males and females, and many of these genes may show significant QTL linkage in only one sex due to limited statistical power. Among genes with a smaller magnitude of divergent expression, the effects are largely independent in males and females; we observe few antagonistic genetic effects such that genes are upregulated in one sex and downregulated in the other (Figure 2A).

We compared ratios of allelic expression in males and females for the 85 genes assayed by pyrosequencing in both sexes. While allelic expression is overall positively correlated between males and females (Figure 3; $\rho = 0.586$, $P < 0.0001$), 37 genes (44%) have

ratios of allelic expression that significantly differ between the sexes ($P < 0.02$, FDR= 0.05). These differences are largely of degree, and the most extreme examples of sexually dimorphic allele-specific expression are genes with strong allelic biases in one sex and little bias in the other. These extreme cases include three genes with modestly but non-significantly elevated expression from the *D. mauritiana* allele in males, but strongly biased expression from the *D. simulans* allele in females (Figure 3). These three genes are located in the same 262 kb chromosomal region (Figure 4), and this clustering of genes with sex-reversed allele-specific expression is significantly different from random expectation (permutation test, $P = 0.0025$). This 262 kb region includes two additional genes that could not be assayed by pyrosequencing in females, but that show significantly greater expression from the *D. mauritiana* allele in males; four of these five genes are expressed exclusively in testes and the fifth is accessory-gland specific in *D. melanogaster*. These observations suggest that *cis*-regulatory control of gene expression across much of this 262 kb region has diverged between these two species in a sex-specific manner.

Among genes with significant QTL associations in at least one sex, we find that both the prevalence and magnitude of sexually dimorphic expression differs between *cis*- and *trans*-regulatory divergence (Figure 2B). First, the proportion of genes with significantly different magnitudes of divergent regulation in males *versus* females (as assessed by non-overlapping 95% confidence intervals) is greater in *cis* (55%) than in *trans* (36%) (Fisher's Exact Test $P_{FET} = 0.0009$). Second, the median absolute value of the difference between males and females in divergent regulation is ~1.4-fold greater among genes regulated in *cis* than in *trans* (Figure 5; Mann-Whitney test $P_{MW} < 0.0001$). Greater sexual dimorphism is associated with larger expression effects (averaged over males and females), although this effect is weaker among divergently *trans*-regulated genes ($\rho = 0.174$, $P < 0.0001$) than it is among divergently *cis*-regulated genes ($\rho = 0.544$, $P < 0.0001$) or among the allele-specific expression ratios obtained from pyrosequencing ($\rho = 0.487$, $P < 0.0001$; Supplemental Figure 3).

In contrast to the homozygous effects of introgressed alleles (Figure 2B), the dominance parameter estimates (Haley and Knott 1992) from the QTL analysis are largely independent between males and females (Figure 2C), indicating that the effects of introgressed alleles are more similar between the sexes when homozygous than when heterozygous. However, as with the homozygous effects, dominance estimates are more disparate between males and females for introgressed *cis*-regulatory variants than factors acting in *trans*. There is a weak but statistically significant positive relationship between male and female dominance parameters among introgressed *trans*-factors ($\rho = 0.089$, $P = 0.008$), in contrast to a weak and marginally significant negative correlation between male and female dominance parameters associated with introgressed *cis*-regulatory divergence ($\rho = -0.22$ $P = 0.046$; Figure 2C). This indicates that, on average, expression effects of *cis*-regulatory divergence in introgression heterozygotes deviate from an additive expectation in the opposite direction in males and females.

Most gene expression traits are oligogenic or polygenic (Brem et al. 2002; Schadt et al. 2003; Brem and Kruglyak 2005; Gibson and Weir 2005); as a consequence, gene expression divergence between the parental *sim*B and *mau12* strains is expected to often result from the combined effects of divergence at multiple loci. Differences between the effects of introgressed factors (relative to *sim*B) and the effects of genome-wide divergence in *mau12* (relative to *sim*B) indicate how the effects of introgressed factors on gene expression are modified by *D. mauritiana* alleles at other loci. We compared the extent of sexually dimorphic expression resulting from introgressed factors with estimates of sexually dimorphic expression between *sim*B and *mau12* at divergently regulated genes. Among genes divergently regulated by *trans*-factors, the effects of the introgressed factors are significantly less sexually dimorphic than the effects of whole-genome divergence ($P_{MW} < 0.0001$; Figure 5). In contrast, the median magnitude of sexual dimorphism among divergently *cis*-regulated genes does not differ between introgression effects and whole-genome species divergence ($P_{MW} = 0.292$). Furthermore, the median magnitude of sexual dimorphism due to whole-genome divergence is similar for genes regulated by introgressed *cis*- and *trans*-factors ($P_{MW} = 0.066$). These observations suggest that *D. mauritiana* alleles outside the introgressed region decouple

*trans*-regulatory effects in males and females more often than effects resulting from *cis*-regulatory divergence.

*Divergent* trans-*regulation is enriched for dominant* D. mauritiana *alleles that cause upregulation*

In addition to the differences in sexual dimorphism, *cis*- and *trans*-regulatory divergence differ substantially in other aspects of their inheritance and evolution between species. First, the magnitude of expression effects, as measured in fold-change, is significantly larger for *cis*-regulatory divergence than *trans*-regulatory divergence (Supplemental Table 5). Second, we observe that *D. mauritiana* alleles of *trans*-regulatory factors tend to up-regulate target genes, while there is no significant bias towards up- or down-regulation as a result of *cis*-regulatory divergence (Table 2). This bias is most pronounced among the largest expression effects, as 42/45 genes divergently *trans*-regulated 2-fold or more are up-regulated by *D. mauritiana* alleles. Third, in both sexes, the effects of *cis*-regulatory divergence are largely additive – the effects on expression in introgression heterozygotes are approximately half the effects in introgression homozygotes – whereas *trans*-acting factors are more often dominant (Figure 6). Furthermore, among genes divergently regulated in *trans,* we observe a sex-by-species bias in dominance relationships; expression in introgression heterozygote males, but not females, is more similar to expression in introgression homozygotes than to *sim*B, indicating that, in males, introgressed *D. mauritiana* alleles of *trans*-regulatory factors are more frequently dominant than their homologous *D. simulans* alleles (Table 3).

Finally, the relationship between the effects of introgressed factors and whole-genome divergence in expression levels differs significantly between *cis*- and *trans*-regulation (Figure 7), indicating that introgressed *cis*- and *trans*-factors are affected differently by *mau12* alleles at loci outside the introgressed region. Homozygous introgressed *cis*-regulatory effects predict well the effects of whole-genome divergence on expression levels at those loci in both sexes ($\rho_{\text{males}} = 0.75$, $\rho_{\text{females}} = 0.85$, $P < 0.0001$). In contrast, the effects of introgressed *trans*-acting factors are significantly poorer predictors of

whole-genome divergence in expression levels. In males, the correlation between homozygous effects due to *trans*-regulatory divergence and interspecific divergence, although positive ($\rho = 0.40$, $P < 0.0001$), is significantly weaker ($P < 0.0001$) than for *cis*-regulatory divergence. In females, there is a significant negative correlation between *trans*-regulatory introgression effects and whole-genome divergence ($\rho = -0.27$, $P < 0.0001$) – on average, *D. mauritiana* alleles at loci outside the introgressions reverse the effects of introgressed *trans*-factors in females.

Previous studies have found that in crosses both within (Gibson et al. 2004) and between (Ranz et al. 2004; Landry et al. 2005; McManus et al. 2010) *Drosophila* species, many genes are expressed in offspring at levels outside the range of both parental genotypes. Such transgressive segregation is consistent with compensatory evolution within species and regulatory incompatibilities among species (Ranz et al. 2003; Ranz et al. 2004; Landry et al. 2005), some of which may contribute to the loss of fitness in hybrids (Michalak and Noor 2003; Michalak and Noor 2004; Good et al. 2010). We refer to those genes expressed in introgression hybrids at levels outside the range of the parental species as *misexpressed.* Substantial majorities of genes in both sexes are misexpressed in introgression hybrids – 58% and 72% of divergently regulated genes in males and females, respectively (this difference in the proportion of misexpression between males and females is statistically significant - $P_{FET} < 0.0001$). We observe an enrichment of misexpressed genes as a result of *trans*-regulatory divergence, although this pattern is only significant in females (Table 4).

*Tissue-specific genes diverge more and are more sexually dimorphic in expression than broadly expressed genes*

The ubiquity of a gene's expression is an important determinant of rates of both sequence and expression evolution. Inducible genes in single-celled organisms (Basehoar et al. 2004; Tirosh et al. 2006), and genes expressed in a restricted set of cell types in multicellular organisms (Duret and Mouchiroud 2000; Larracuente et al. 2008) evolve significantly more rapidly than ubiquitously expressed genes. Consistent with these

earlier findings, breadth of expression across tissues and organs has a significant impact on the patterns of divergence and sex-biased expression in our data. The magnitude of the effects of introgressed factors on gene expression, whole-genome expression divergence between *sim*B and *mau12*, the magnitude of allele-specific expression in introgression heterozygotes, and the difference in these effects between males and females are all larger among tissue-specific genes than broadly expressed genes (Supplemental Table 6); these results generally hold for both *cis*- and *trans*-regulatory divergence. As whole animals were used for expression analysis, the expression effects among tissue-specific genes are almost certainly underestimated (Chintapalli et al. 2007), suggesting that the effects of tissue-breadth are even more pronounced than these tallies suggest.

*Co-regulated genes with immune function are misexpressed in introgression females*

There is a significant enrichment of genes associated with the immune response in *D. melanogaster* (as annotated on FlyBase) among transcripts divergently regulated in females ($P_{FET} < 0.0001$), but not in males ($P_{FET} = 0.6$). In particular, among the 25 divergently regulated genes that are upregulated at least 2-fold or more in homozygous introgression females, ten have annotated immune function and QTL mapping localizes the causative factor to one of two adjacent markers (Supplemental Table 7). Together, these results are consistent with an introgressed functionally divergent *D. mauritiana* allele of a *trans*-regulatory factor that affects the expression of multiple effectors of the humoral immune response distributed throughout the genome.

The expression of these ten immunity genes is positively correlated between the sexes in introgression genotypes ($\rho = 0.78$, $P = 0.008$; Supplemental Figure 4), indicating that, in a mostly *D. simulans* genetic background, the *D. mauritiana* allele of this *trans*-acting factor has similar effects in males and females. In contrast, relative expression of these genes is uncorrelated between *mau12* males and females ($P = 0.32$); males show variable expression, and females show $\geq$ 2-fold downregulation and misexpression. These observations are consistent with a model whereby *D. mauritiana* alleles at one or more

loci outside the introgressed region decouple the effects of this *trans*-regulator in males and females, mainly by reversing its effects in females.

**Discussion**

We used microarray analysis of introgression hybrids between *D. mauritiana* and *D. simulans* to sample the genetic basis for genome-wide differences in gene expression between these two species. Below we describe how the evolutionary histories of these species and our approach circumscribe our study before turning to the evolutionary conclusions that we draw from our genetic analyses.

First, *D. simulans* and *D. mauritiana* diverged ~242,000 years ago and differ at 1.6% of sites across the euchromatic portion of the genome (Garrigan et al. 2012), and by at least 1000 structural differences totaling 1Mb of euchromatic DNA (Langley et al. 2012). Both species have large effective population sizes and high levels of nucleotide polymorphism (Kliman and Hey 1993; Nolte et al. 2013). Given the large effective population sizes of both species and the recency of their divergence, some unknown fraction of the genetic factors that we identify are likely polymorphisms segregating in one or both species, rather than fixed differences.

Second, *D. simulans* and *D. mauritiana* are separated by multiple systems of reproductive isolation, including premating (Coyne 1992; Coyne and Charlesworth 1997), postmating-prezygotic (Price et al. 2000), and intrinsic postzygotic isolation (Lachaise et al. 1986). It has been estimated that ~15 loci across the genome can confer complete male sterility and 2-3 loci cause lethality when introgressed from *D. mauritiana* into *D. simulans* (Tao et al. 2003; Cattani and Presgraves 2009). Multiple mtDNA haplotypes have recently introgressed from *D. simulans* into *D. mauritiana* (Ballard 2000; Nunes et al. 2010; Garrigan et al. 2012), and 2-5% of 5kb genomic segments have evolutionary histories consistent with recent gene flow between these two species (Garrigan et al. 2012). Thus, there is the opportunity for gene flow between these two species, but significant barriers

to gene flow also exist in the form of both a few major and presumably many more minor hybrid incompatibilities (True et al. 1996). Due to the accumulation of intrinsic postzygotic incompatibilities, we expect that many gene expression phenotypes associated with introgressed factors are deleterious; however, we do not know the fitness effects associated with the observed expression phenotypes. Nonetheless, in view of the reduced opportunity for selection to act in hybrids relative to the parental species, we assume that introgression hybrids are more likely to have deleterious patterns of gene expression than either of the pure species genotypes.

Third, we observe that ~90% of divergently regulated genes change expression as a result of species divergence at *trans*-factors (Table 1). Most previous studies in *Drosophila* have inferred that *cis*-regulatory divergence can account for at least 50% of differences in gene expression both within (Hughes et al. 2006; Genissel et al. 2008; Lemos et al. 2008a) and between species (Wittkopp et al. 2004; Wittkopp et al. 2008; McManus et al. 2010). The large proportion of *trans*-divergence we detect here can likely be attributed to our use of homozygous introgression hybrids, as opposed to assaying $F_1$ progeny, for two reasons. First, our introgression approach sampled regulatory divergence across 9.3 Mb and estimated the effects of this divergence on genome-wide gene expression. Due to the polygenic nature of gene expression (Brem et al. 2002; Schadt et al. 2003; Brem and Kruglyak 2005; Gibson and Weir 2005), some differences in gene expression between these species result from divergence at multiple *trans*-acting loci. Because some genes divergently regulated by an introgressed *trans*-factor are also regulated by divergence at loci not captured by these introgressions, this approach overestimates the proportion of genes genome-wide that are divergently regulated in *trans*. Second, regulatory alleles that act recessively will be masked in $F_1$ hybrids. Previous studies (Lemos et al. 2008a; McManus et al. 2010; Zhang et al. 2011; Gruber et al. 2012) and our results suggest that recessive alleles more frequently underlie *trans*-regulatory divergence (Table 4); $F_1$ analyses therefore likely underestimate divergent *trans*-regulation. A recent mutagenesis study in *S. cereviseae* is also broadly consistent with the idea that genome-wide heterozygosity may mask *trans*-regulatory variation; Gruber *et al* found that >80% of *de novo* mutations assayed in a common genetic background acted in *trans* to modulate

reporter gene expression, and these *trans*-mutations were masked in heterozygous diploids (Gruber et al. 2012).

Finally, we studied a single autosomal region for effects on genome-wide gene expression. It is therefore important to consider which results are likely to generally hold for regulatory divergence between *D. simulans* and *D. mauritiana*, and which may be idiosyncratic to the evolutionary history of this particular region of the genome. We assume that the majority of *cis*-regulatory divergence acts gene-specifically, excepting the candidate region highlighted in Figure 5. Patterns associated with divergence in *cis* therefore result from many independent regulatory substitutions, and therefore are likely to generally apply to autosomal, euchromatic *cis*-regulatory divergence between these species. In both sexes, each of the ten genetic markers (Figure 1) showed linkage to multiple *trans*-regulated expression phenotypes, indicating that, at a minimum, ten divergent factors are required to account for the *trans*-regulatory divergence we observe. Patterns that we observe among *trans*-regulated expression phenotypes linked to all markers are the result of divergence at multiple regulatory factors, and are therefore also likely to reveal general features of regulatory divergence between these two species. In contrast, patterns that are driven by expression phenotypes linked to only one marker are more likely to be idiosyncratic consequences of the specific mutations captured by these introgressions. Across both sexes, 67% of genes up-regulated more than 1.5-fold in *trans* show linkage to the same marker, and many of these genes have annotated immune function, suggesting that the large fraction of up-regulation in these genotypes (Table 2) and the enrichment of strongly up-regulated genes with immune function (Supplemental Table 7) may be due to a single introgressed *trans*-regulatory factor of large effect. All other patterns we describe are more likely to be general features of regulatory divergence in *Drosophila*.

Although a bias towards up-regulation in *trans* has been observed among *de novo* regulatory mutations in *Saccharomyces cereviseae* (Gruber et al. 2012), studies in *Drosophila* have found a significant excess of genes down-regulated in interspecific $F_1$ hybrids relative to both parent species (Michalak and Noor 2003; Ranz et al. 2004;

Moehring et al. 2007; McManus et al. 2010). However, these studies profiled sterile individuals with sterility phenotypes that range from post-meiotic disruption of gametogenesis (Kulathinal and Singh 1998) to largely agametic (Sturtevant 1920), and the large number of underexpressed genes results at least in part from assaying whole animals lacking germline cells, compared to fertile individuals from the parental species (Ranz et al. 2004; McManus et al. 2010). Both sexes of the introgression hybrids used here are fertile, and so gross differences in tissue composition between genotypes are not expected. As mentioned above, the up-regulation in *trans* we detect may be attributable to a single large-effect factor; it therefore remains unclear if biases towards up- or down-regulation are in fact common features of gene misregulation in *Drosophila* hybrids.

*The evolution of sexually dimorphic gene expression*

Assaying gene expression in both males and females allowed us to identify sex-biased and sex-specific effects of regulatory divergence. We detect significantly more divergently regulated genes in males than in females despite the fact that the false discovery rate is slightly higher in females (see Methods). An excess of *cis*-eQTL in males was also detected in a North Carolina population of *D. melanogaster* (Massouras et al. 2012). One possible explanation for these observations is that global gene expression in *Drosophila* males is simply more sensitive than expression in females to genetic variation and divergence. For example, genetic perturbations affecting heterochromatin have more severe effects on genome-wide expression in males than females (Liu et al. 2005; Deng et al. 2009), which may reflect interactions with male-specific factors that affect both euchromatic and heterochromatic gene regulation, such as the Y chromosome (Lemos et al. 2008b), or the somatic dosage compensation complex (Deng et al. 2009). However, we caution that greater statistical power associated with the microarray experiments in males, *e.g.*, due to greater technical variation or sensitivity to random environmental effects in females, could potentially contribute to the excess of divergently regulated genes detected in males.

It has been proposed that the evolution of sexually dimorphic gene expression is driven in part by selection to resolve sexually antagonistic fitness variation (Rice 1984). This model assumes that mutations are generally less sexually dimorphic than required for optimal fitness in both sexes, in contrast to a model where most mutations have disparate effects in males and females, and subsequent evolution restores fitness to the disfavored sex via *reducing* sexual dimorphism in expression. Mutation accumulation experiments in animals have measured the effects of *de novo* mutations on gene expression in one sex only (Denver et al. 2005; Rifkin et al. 2005), so the degree of sexual dimorphism among new mutations affecting gene expression levels is currently unknown. We observe that, among genes regulated by divergent *trans*-factors, the effects of introgressed alleles on gene expression are more similar between the males and females than the effects of genome-wide divergence between *sim*B and *mau12*, indicating that *D. mauritiana* alleles elsewhere in the genome increase sexual dimorphism in expression relative to the introgressed *trans*-factors. Assuming that reduced dimorphism in some expression phenotypes due to introgressed regulatory factors confers decreased fitness relative to the pure species genotypes, this pattern supports the scenario whereby compensatory evolution increases the sexual dimorphism of individual mutations and suggests the existence of the modifier alleles predicted by Rice's model (Rice 1984).

The median homozygous effect of introgressed *cis*-regulatory sequences is 40% more sexually dimorphic than the median effect of introgressed *trans*-regulatory factors (Figure 5), and the effects of *cis*-regulatory divergence in males and females are similar in the *sim*B and *mau12* backgrounds (Figure 5, Supplemental Figure 5). This indicates that, while *mau12* alleles at loci outside the introgressions modulate the effects of introgressed *trans*-factors to increase sex differences in expression, such modulation is, on average, less frequent for *cis*-regulatory divergence. Case-studies have repeatedly identified that divergence at *cis*-regulatory elements is responsible for the evolution of sex-specific patterns of gene expression (Gompel et al. 2005; Jeong et al. 2006; Williams et al. 2008); and a population genomic study found that 75% of *cis*-eQTL are sex-specific within a single *D. melanogaster* population (Massouras et al. 2012). Together with these studies, our results suggest that disparity in expression between the sexes may be a general

feature of *cis*-regulatory control, and that this disparity includes overall expression levels, in addition to the timing and spatial patterns of gene expression through development. If different expression optima in males and females are common, this may be an important pleiotropic constraint that is alleviated by *cis*-regulatory evolution (Stern 2000).

The enrichment of sexually dimorphic gene expression associated with *cis*-regulation could conceivably result from either molecular mechanisms of gene regulation or evolutionary processes. In *Drosophila,* global differences between males and females in the balance of euchromatin and heterochromatin (Deng et al. 2009) and the distribution of covalent histone modifications (Lucchesi et al. 2005) may give males and females distinct chromatin states across the genome, which could enrich sexually dimorphic effects among *cis*-regulatory mutations. An alternate, but not mutually exclusive, possibility is that *cis*-regulatory mutations that affect sexually dimorphic expression are more likely to be tightly linked to sexually antagonistic protein-coding mutations located in the same gene, potentially facilitating the spread of both mutations through a population (Rice 1984).

*Dominance and the evolution of gene expression*

Assaying gene expression in homozygous and heterozygous introgression genotypes allowed us to estimate the dominance of divergent regulatory factors. We observe that, in contrast to the additive effects of introgressed factors, dominance effects are independent between males and females, and are even weakly negatively correlated among *cis*-regulatory divergence (Figure 2C). Furthermore, *D. mauritiana trans*-regulatory alleles are predominantly dominant in males only (Table 3). These observations have important implications for our understanding of sexually antagonistic fitness variation, as the dominance of sexually antagonistic mutations is a critical parameter determining their fate in populations. Sex-specific dominance resulting from X-chromosome hemizygosity in males allows X-linked sexually antagonistic alleles to segregate at higher frequencies than equivalent autosomal alleles that have similar dominance effects between males and females (Rice 1984). However, we observe extensive sex-specific dominance in

expression effects associated with introgressed autosomal regulatory factors. If sex-specific dominance is common, then the fate of sexually antagonistic alleles within populations may be largely determined by the particular combination of sex-specific fitness effects and dominance relationships associated with these mutations, and sexually antagonistic fitness variation may not be restricted to the X chromosome (Fry 2010).

The dominance of gene expression changes differs significantly between divergent *cis*- and *trans*-regulation. Divergent *trans*-regulation is enriched for dominant or recessive alleles, while the effects of *cis*-regulatory divergence are intermediate in heterozygous genotypes (Figure 6, Table 3). Additivity has been previously documented for *cis*-regulatory variants (Wray 2007; Lemos et al. 2008a; McManus et al. 2010; Gruber et al. 2012), and is expected in the absence of transvection due to independent transcription of homologous alleles in a diploid heterozygote (Wray 2007). As additive effects expose rare alleles to selection, this may cause fewer advantageous *cis*-regulatory alleles to be lost to drift, potentially enriching their fixation relative to their mutational origination as compared to *trans*-acting mutations (Wray 2007). Additionally, additivity may enrich *cis*-regulatory variants for mutations with heterozygote advantage and influence levels of standing *cis*-regulatory genetic variation (Sellis et al. 2011).

*The contributions of* cis- *and* trans-*regulatory divergence to the evolution of gene expression*

The average fold-change resulting from *cis*-regulatory divergence identified here is significantly larger than those resulting from *trans*-divergence (Supplemental Table 5), consistent with what appears to be a general pattern of larger expression effects associated with *cis*-regulatory variation (Brem et al. 2002; Schadt et al. 2003; Hughes et al. 2006; Lemos et al. 2008a; McManus et al. 2010; Gruber et al. 2012). The effects of divergent *trans*-regulatory factors are weakly correlated (in males) or anti-correlated (in females) with overall expression differences between *sim*B and *mau12*, while the effects of *cis*-regulatory divergence are positively correlated with interspecific expression differences (Figure 7), resulting in an excess of gene misexpression due to divergent

*trans*-regulation (Table 4). In our experiments, divergent regulation in both *cis* and *trans* results from interspecific interactions between alleles that modulate gene expression (*D. simulans trans*-factors with *D. mauritiana cis*-regulatory sequences and *D. mauritiana trans*-factors with *D. simulans cis*-regulatory sequences, respectively). However, only divergent *trans*-regulation is affected by interactions between *trans*-factors from both species. Interspecific interactions between *trans*-factors, then, could be responsible for the observed misexpression bias. If true, this suggests that misexpression rarely results from direct interactions between *trans*-factors and *cis*-regulatory sequences, but rather as a downstream consequence of interspecific interactions between alleles at two *trans*-regulatory factors, or between alleles at loci whose effects on gene expression are indirect (Yvert et al. 2003).

Finally, our results have implications for the evolution of gene expression phenotypes and the accumulation of incompatibilities that contribute to or follow reproductive isolation. For gene expression levels that are under stabilizing selection in both species – which is likely the majority (Rifkin et al. 2003; Denver et al. 2005; Lemos et al. 2005; Ronald and Akey 2007) – expression phenotypes that are deleterious in the introgression hybrid relative to both parental species will be enriched among misexpressed genes, where expression in the hybrid is outside the range of the parents. Such expression incompatibilities will therefore be more common among *trans*-regulatory divergences, and as described above, these may result mostly from *trans*-*trans* interactions. This suggests that evolutionary turnover of *cis*-regulatory DNA and the proteins that interact with these sequences may contribute less to the evolution of incompatibilities, at least for the expression of euchromatic single-copy genes. Additionally, if a significant fraction of regulatory mutations select for secondary mutations that modify regulatory effects in a sex-specific manner, then sex-specific misexpression may contribute to a loss of fitness in hybrids.

**Methods**

*Drosophila genetics and husbandry*

The construction of the introgression genotypes used here was originally described in (Tao et al. 2003). Stocks of a *D. mauritiana* $w^-$ strain (*mau12*) carrying a co-dominant visible marker (P[$w^+$]) on the 3rd chromosome were crossed to a $w^-$ strain of *D. simulans*, and hybrid females carrying *D. mauritiana* material marked by P[$w^+$] were backcrossed to *D. simulans* $w^-$ males for four generations. Introgression lines were then crossed to a $w^-$ genotype of *D. simulans* (referred to hereafter as *sim*B) with a recessively marked 2nd chromosome, and 3rd chromosomes carrying introgressed segments were passaged through males to ensure that the X and 2nd chromosomes were derived entirely from *sim*B. To eliminate mutations that may have accumulated in these lines since their construction (*e.g.,* (Denver et al. 2005; Rifkin et al. 2005; Landry et al. 2007)), the introgressed *D. mauritiana* segments were re-extracted into the *sim*B background using the crossing scheme shown in Supplemental Figure 6. In the final generation of the re-extraction, single males and females were paired to create sublines of each original introgression genotype. To confirm that the introgressions remained intact following two generations of recombination in females, all sublines were genotyped at markers originally used to identify the limits of the introgressions (Tao et al. 2003); following confirmation, five sublines were selected and pooled to regenerate the introgression lines. Seven lines carrying introgressed *D. mauritiana* genomic segments in cytological divisions 66B-71B on chromosome arm 3L were used for the current experiments. Gene expression was assayed in homozygous introgression genotypes, heterozygous introgression genotypes (introgression/*sim*B), and the parental *sim*B and *mau12* strains, for a total of 16 genotypes.

All flies used for RNA extraction were reared on standard cornmeal molasses media in glass vials at 25C on a 12:12 light:dark cycle. Culture density was controlled by combining 15 males with 15 females per vial for homozygous introgression genotypes, and 10 males with 10 females for the *sim*B, *mau12*, and heterozygous introgression genotypes. Flies for RNA extraction were reared in three independent cohorts. Adults were collected as virgins and the sexes were aged separately for 1-2 days before flash-

freezing in liquid nitrogen and storage at -80C. All flies were frozen at the same time of day to control for circadian effects on gene expression. Heterozygous introgression genotypes were generated from both reciprocal crosses, *i.e.,* virgin *sim*B females were crossed to introgression line males and virgin introgression line females were crossed to *sim*B males, and equal amounts of RNA extracted from the offspring of reciprocal crosses were pooled for microarray hybridization. This approach averages over any potential parent-of-origin effects on expression and thus such effects can neither be detected nor confound our results; in any case, there is little evidence for imprinting or parent-of-origin effects on gene expression in adult *Drosophila* (Coolon et al. 2012).

*Microarray and pyrosequencing methods*

Microarray construction, RNA extraction, cDNA synthesis and hybridization were done following standard protocols. Two-color competitive hybridizations followed a modified loop design (Supplemental Figure 7) that minimizes the distance between any two genotypes (Townsend 2003). Allele-specific expression was assayed by pyrosequencing at 90 candidate genes identified from the microarrays as potentially harboring *cis*-regulatory variants (Supplemental Figure 8, Supplemental Table 1). Detailed microarray and pyrosequencing methods are available as Supplemental Material.

*Statistical analyses*

Microarray log2 expression ratios were analyzed with an empirical Bayes linear model (Smyth 2004) and relative expression levels (log2 fold-change) were estimated for each homozygous and heterozygous introgression genotype, *sim*B, and *mau12*, setting expression levels in *sim*B = 0. Estimates of gene expression levels obtained from the linear model were then used for QTL analysis. Expression estimates from *mau12* were excluded from the QTL analysis, as this strain differs in genetic background from the *sim*B and introgression lines. Expression levels in *mau12* were used in post-hoc comparisons to infer patterns of gene expression evolution and misexpression.

Genotype data for QTL mapping was collected at markers across the introgressed region (Figure 1). Marker regression (Haley and Knott 1992) was used to detect associations between marker genotype (mau/mau, mau/sim, or sim/sim) and gene expression level (log2 fold-change relative to *sim*B). Significance was determined by a $\chi^2$ test and the effects of introgression genotypes on expression phenotypes are reported as the additive (*a*) and dominance (*d*) parameters estimated from the Haley-Knott regression. In this model, expression in homozygous introgression genotypes is estimated by 2*a* and expression in heterozygous introgression genotypes by $a + d$. QTL analyses were done using R/qtl v1.23 (Broman et al. 2003).

The *P*-value cutoff for assigning a statistically significant association between expression phenotypes and genetic markers was obtained by setting the false discovery rate (FDR)(Benjamini and Hochberg 1995) to 0.02 in males, which corresponds to a nominal $\chi^2$ *P*-value of 0.00021. This *P*-value cutoff was then used for both the male and female experiments, resulting in an FDR of 0.03 in females. This FDR was calculated using all linkage tests at all markers, but we considered only the marker with the best fit for each gene expression phenotype. This approach is very conservative with respect to false positives, as shown in Q-Q plots of the QTL linkage *P*-values (Supplemental Figure 9). A common *P*-value cutoff and slightly different FDR rates across the sexes was deemed preferable to controlling the FDR to a common value in both males and females, as an FDR of 0.02 in females corresponds to a nominal *P*-value of 0.000065, which reduces the number of observed significant associations in females by half. Our decision to set a common *P*-value cutoff is therefore conservative with regard to the excess in the number of significant associations detected in males.

Normalized log2-transformed relative expression measures from pyrosequencing were analyzed in two steps. First, a mixed linear model including main effects of sex, strain, reciprocal cross, and all two-way interactions were fit for all genes separately, excepting four genes assayed using only one genotype, for which the model included sex, reciprocal cross, and their interaction. The results of these models were examined and effects of strain and cross and their interactions were dropped if they were not significant. Based

on this approach, one of three statistical models was used for each gene (see Supplemental Table 8):

1) log2 normalized ratio of allelic expression ~ sex + strain + ε (4 genes)

2) log2 normalized ratio of allelic expression ~ sex + cross + sex * cross + ε (5 genes)

3) log2 normalized ratio of allelic expression ~ sex + ε (81 genes)

All models used type III sums of squares and were run using the GLM procedure in SAS statistical software (version 10).

*FlyAtlas analyses*

Gene expression estimates from 20 tissues dissected from larval and adult *D. melanogaster* were compiled from FlyAtlas (Chintapalli et al. 2007). Affymetrix microarray probe sets with multiple matches to the genome were excluded from the analysis, and among genes with multiple probe sets, the probe set with the highest mean signal intensity across samples was selected. Signal intensities at probe sets with absent calls were set to one, and all intensity values were log2 transformed. Mean expression values were then calculated from four replicate arrays. Tissue-specificity was calculated as the $\tau$ statistic (Yanai et al. 2005).

All statistical analyses except for the pyrosequencing models were done in R (v2.14.1) (Team 2011). Microarray analyses used the *limma* package (v3.10.3); standardized major axis regression was used for bivariate regression analysis with the *smatr* package (v3.2.4).

## Data Access

Microarray data have been deposited in the NCBI GEO database under the series GSE44329. Pyrosequencing data are included in Supplemental Table 8.


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## Author Contributions

CDM, JDC, and PJW conceived and designed the experiments; CDM and JDC performed the experiments; CDM and JDC analyzed the data; CDM, DLH, and PJW contributed reagents/materials/analysis tools; CDM wrote the paper.

## Disclosure Declaration

The authors declare no conflicts of interest

## Figure Legends

Figure 1. Approach used to map factors within the introgressed region that affect gene expression. (A) Genomic regions marked with a visible transgene (P[$w^+$]) were introgressed from *D. mauritiana* into a *D. simulans* background. Bars indicate the sex chromosomes (left) and the two major autosomes. (B) Gene expression was assayed in adult males and females from seven genotypes carrying overlapping introgressions on chromosome arm 3L. Horizontal black bars indicate the extent of the introgressed

segments, and vertical lines denote the location of genotyping markers. Expression levels in the seven homozygous introgression lines are shown to the right for an exemplar gene (*Est-6*). (C) QTL mapping identified marker loci within the introgressions where the *D. mauritiana* allele has a detectable effect on gene expression relative to the background *D. simulans* strain.

Figure 2. Introgression effects on expression in males and females for 964 genes divergently regulated in at least one sex and expressed in both sexes. (A). Homozygous introgression effects (twice the additive effect estimated from Haley-Knott regression) relative to the parental *D. simulans* strain. Symbols indicate whether gene expression phenotypes show significant eQTL in only one sex (sex-spp) or both males and females (both linked), and among the latter group, whether the linked genetic marker in males and females is shared (the same or adjacent markers) or not (disparate markers). For genes with shared markers between the sexes, expression effects are strongly positively correlated (green dashed line, slope = 1.15, $\rho = 0.90$, $P < 0.0001$). (B). Homozygous introgression effects in males versus females for *cis*- and *trans*-regulated genes. (C). Dominance parameters for expression effects in males and females, for *cis*- and *trans*-regulated genes. Dominance parameters are weakly positively correlated for trans-regulated genes ($\rho = 0.089$, $P = 0.008$), and weakly negatively correlated among cis-regulated genes ($\rho = -0.22$, $P = 0.046$).

Figure 3. The ratio of expression from the *D. mauritiana* and *D. simulans* alleles in introgression heterozygotes is plotted for females versus males for 85 genes assayed by pyrosequencing. Three genes located in a region with sex-reversed allele-specific expression are highlighted in red. Error bars indicate 95% confidence limits.

Figure 4. Allele-specific expression among genes assayed by pyrosequencing within the introgressed region. (A) Log2 ratios of allelic expression (*D. mauritiana*/*D. simulans*) in males and females. Trend lines were obtained by loess smoothing using a span of 0.2. The candidate region containing local sexually dimorphic cis-regulatory effects is highlighted in grey. (B) Differences in allelic expression between males and females as a

function of location. Trend line was obtained by loess smoothing using a span of 0.2. Candidate region with sexually dimorphic cis-regulatory effects is apparent as a net excess of *D. mauritiana* allelic expression in males, due to strong overexpression of the *D. simulans* allele in females.

Figure 5. Introgressed *cis*-regulatory divergence generates greater sexual dimorphism in expression than introgressed *trans*-regulatory factors. White boxes show the distribution of the absolute differences between males and females in the effects of introgressed factors measured by microarray, as well as the absolute difference between the sexes in allelic expression measured by pyrosequencing. Grey boxes show the distribution of the absolute differences in expression between males and females resulting from whole-genome divergence between the parental *sim*B and *mau12* strains for the same genes.

Figure 6. Differential inheritance of gene expression phenotypes in *cis* versus in *trans*. *Cis*-regulation affects expression additively (males: slope = 1.97, $\rho = 0.958$; females: slope = 2.46, $\rho = 0.817$, $P < 0.0001$ in both sexes), while trans-regulation is associated with more dominant *D. mauritiana* alleles (males: slope = 1.26, $\rho = 0.900$; females: slope = 1.51, $\rho = 0.809$, $P < 0.0001$ in both sexes).

Figure 7. Differential evolution of gene expression phenotypes in *cis* versus in *trans*. In both sexes, the effects of introgressed *cis*-regulatory divergence are predictive of whole-genome divergence (males: slope = 1.07, $\rho = 0.75$; females: slope = 0.86, $\rho = 0.85$, $P < 0.0001$ in both sexes). In contrast, the effects of introgressed *trans*-regulatory factors in males are poorer predictors of whole-genome divergence (slope = 0.77, $\rho = 0.40$, $P < 0.0001$), implicating modifier alleles elsewhere in the genome. In females, the effects of introgressed *trans*-regulatory factors are, on average, reversed by alleles elsewhere in the genome in females (slope = -0.79, $\rho = -0.27$, $P < 0.0001$). The blue line has a slope of one.

**Tables**

Table 1. Number of detectably expressed and divergently regulated genes.

Table 2. *Trans*-regulatory divergence is biased towards up-regulation.

Table 3. Dominance patterns by sex, genetic control, and direction of regulation.

Table 4. Gene misexpression results more frequently from divergent *trans*-regulation than *cis.*

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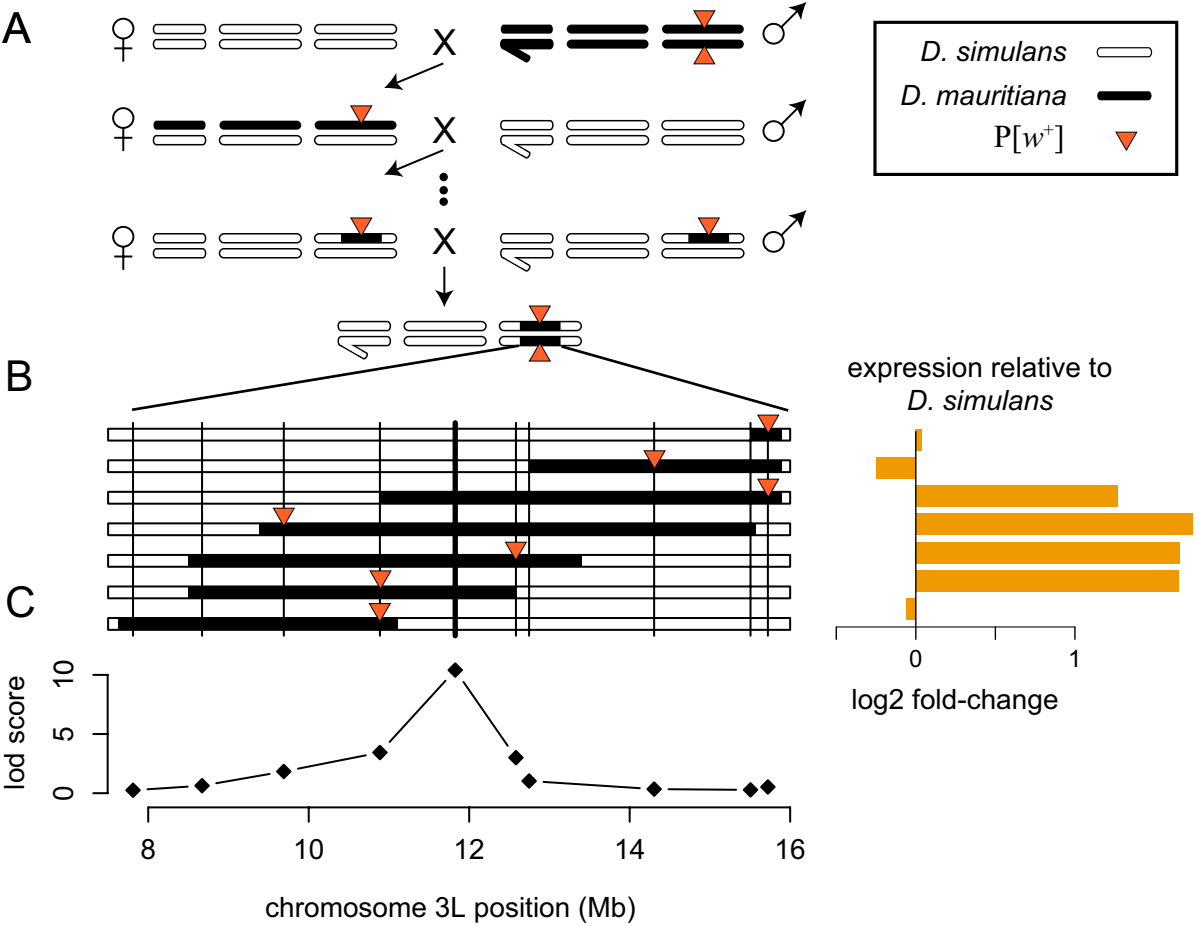
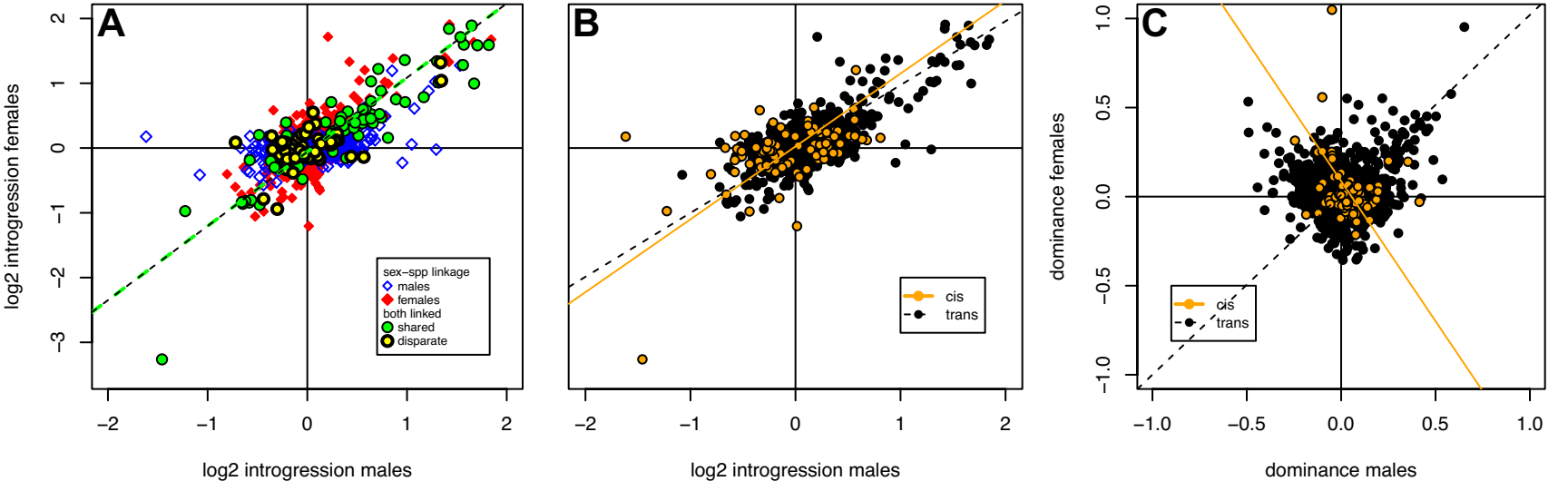
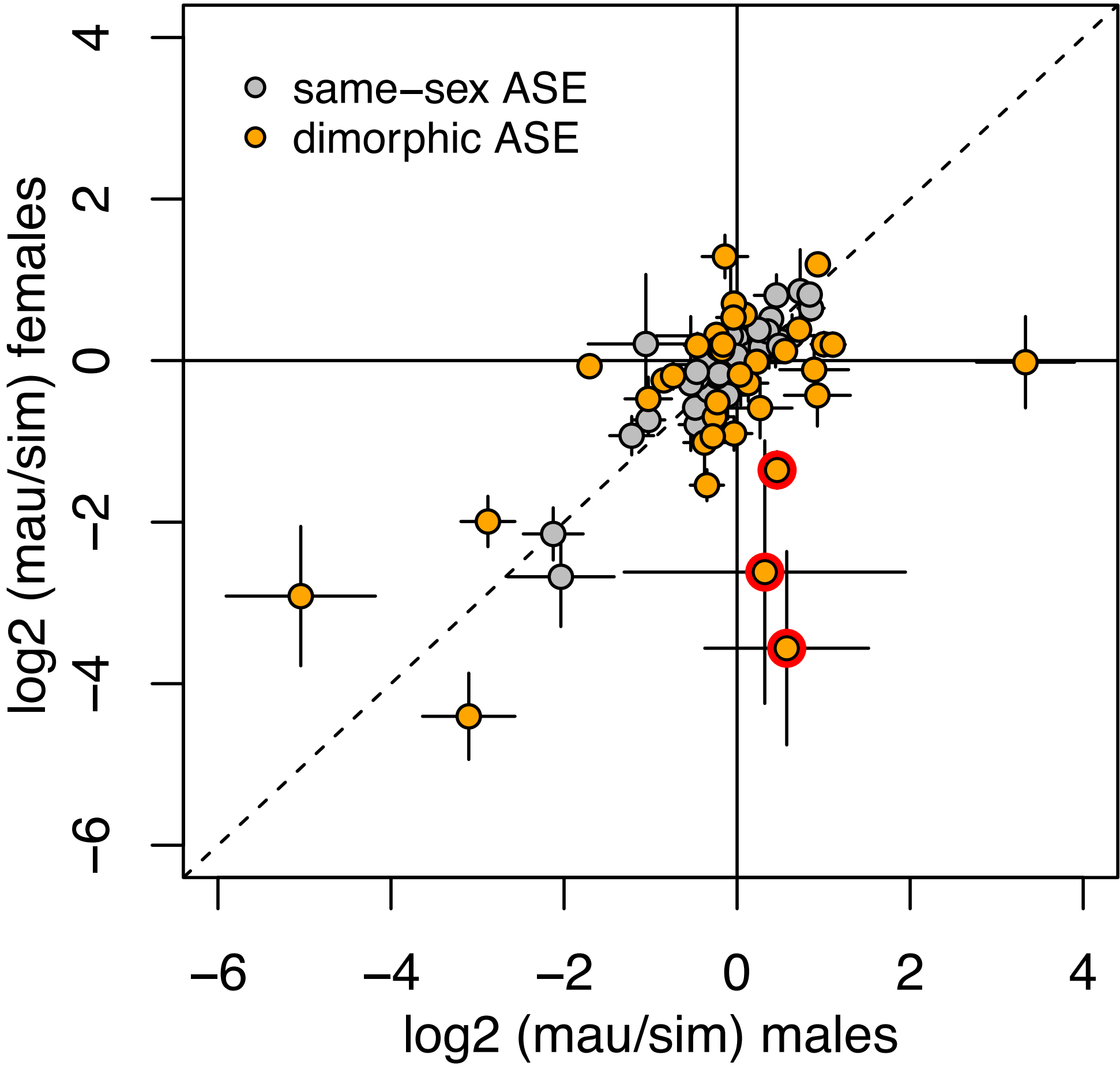
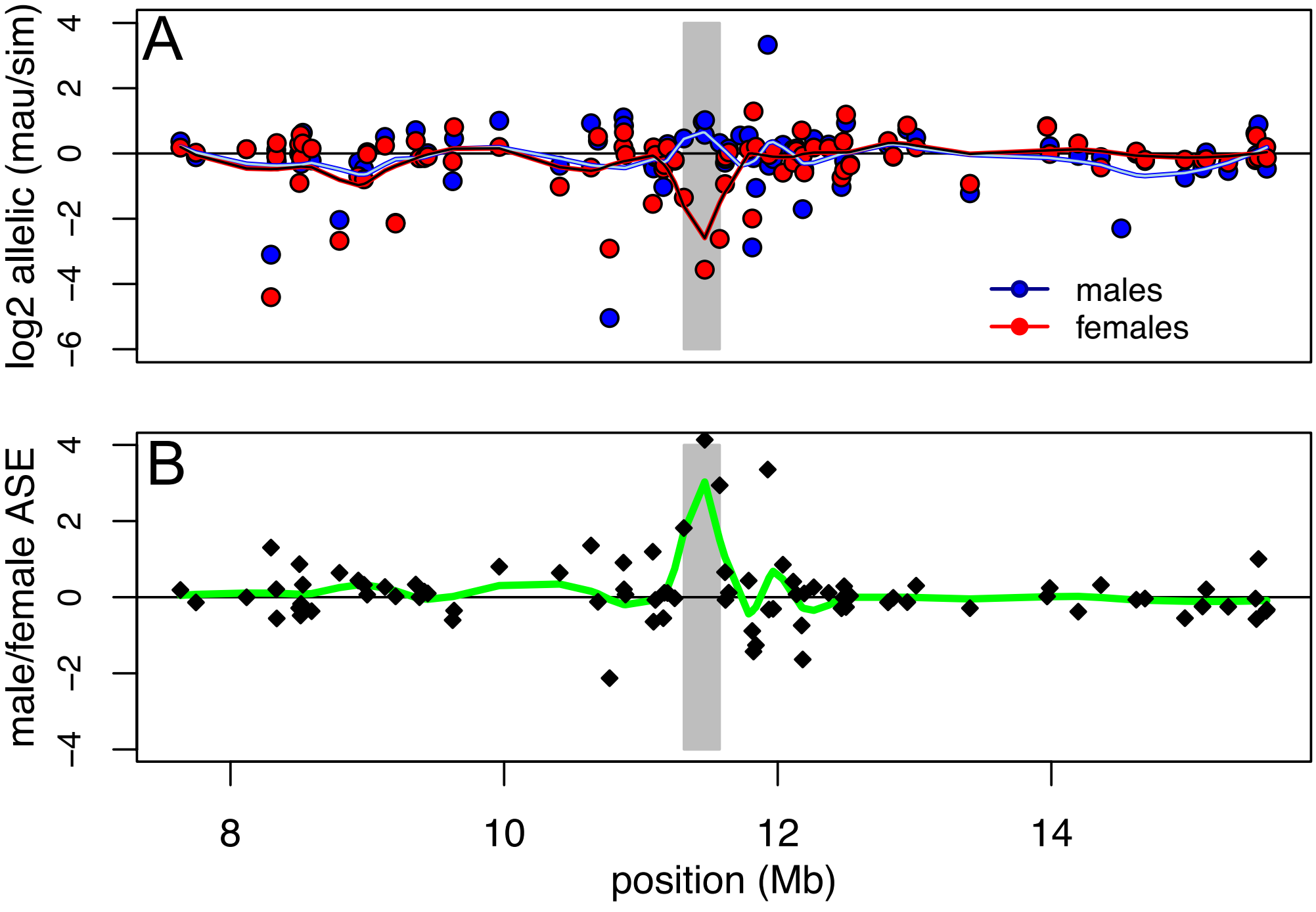
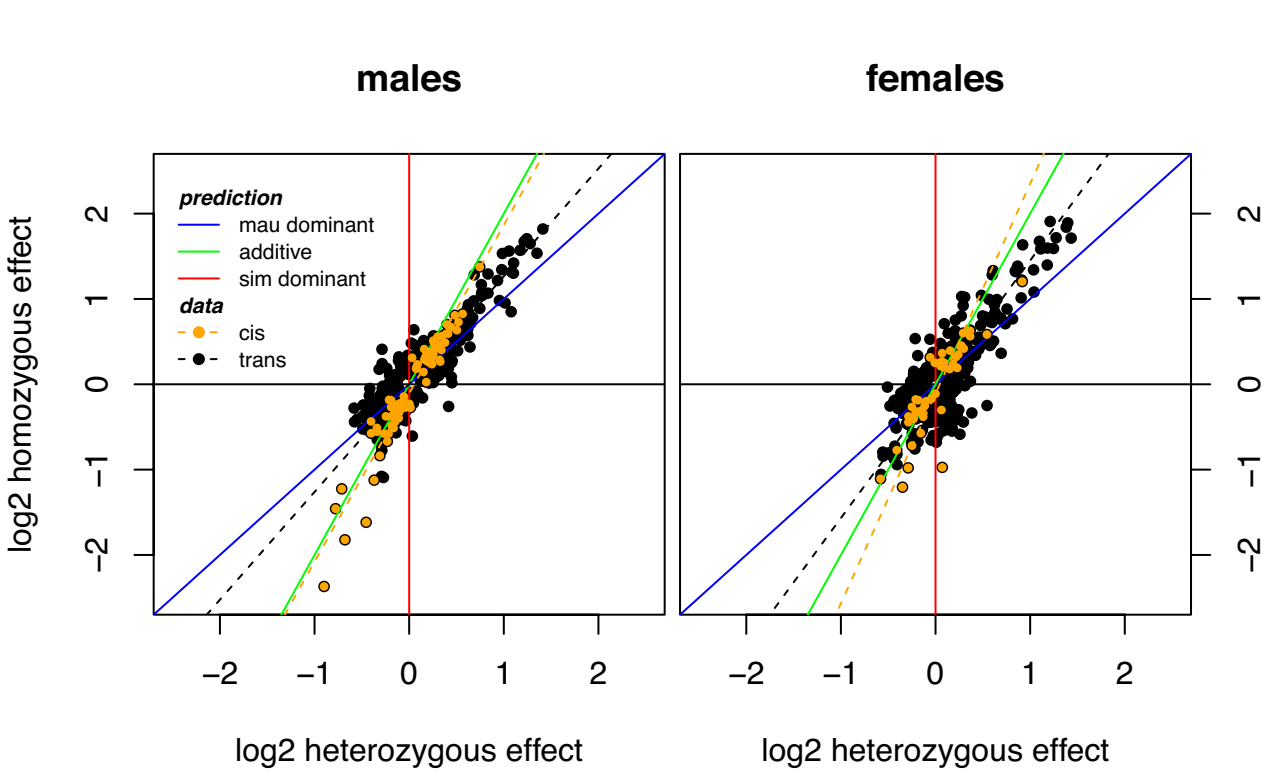

A
B
C
D. simulans
D. mauritiana
P[w+]
expression relative to
D. simulans
0
1
log2 fold-change
lod score
0
5
10
8
10
12
14
16
chromosome 3L position (Mb)


A
log2 introgression females
log2 introgression males
sex−spp linkage
males
females
both linked
shared
disparate
B
log2 introgression males
cis
trans
C
dominance females
dominance males
cis
trans


same−sex ASE
dimorphic ASE
log2 (mau/sim) females
log2 (mau/sim) males
4
2
0
−2
−4
−6
−6
−4
−2
0
2
4


A
log2 allelic (mau/sim)
4
2
0
−2
−4
−6
males
females
B
male/female ASE
4
2
0
−2
−4
8
10
12
14
position (Mb)

introgression effects
whole genome divergence
l male - female l
0
1
2
3
4
trans
trans
cis
cis
pyro


males
females
prediction
mau dominant
additive
sim dominant
data
cis
trans
log2 homozygous effect
log2 heterozygous effect
log2 heterozygous effect
−2
−1
0
1
2

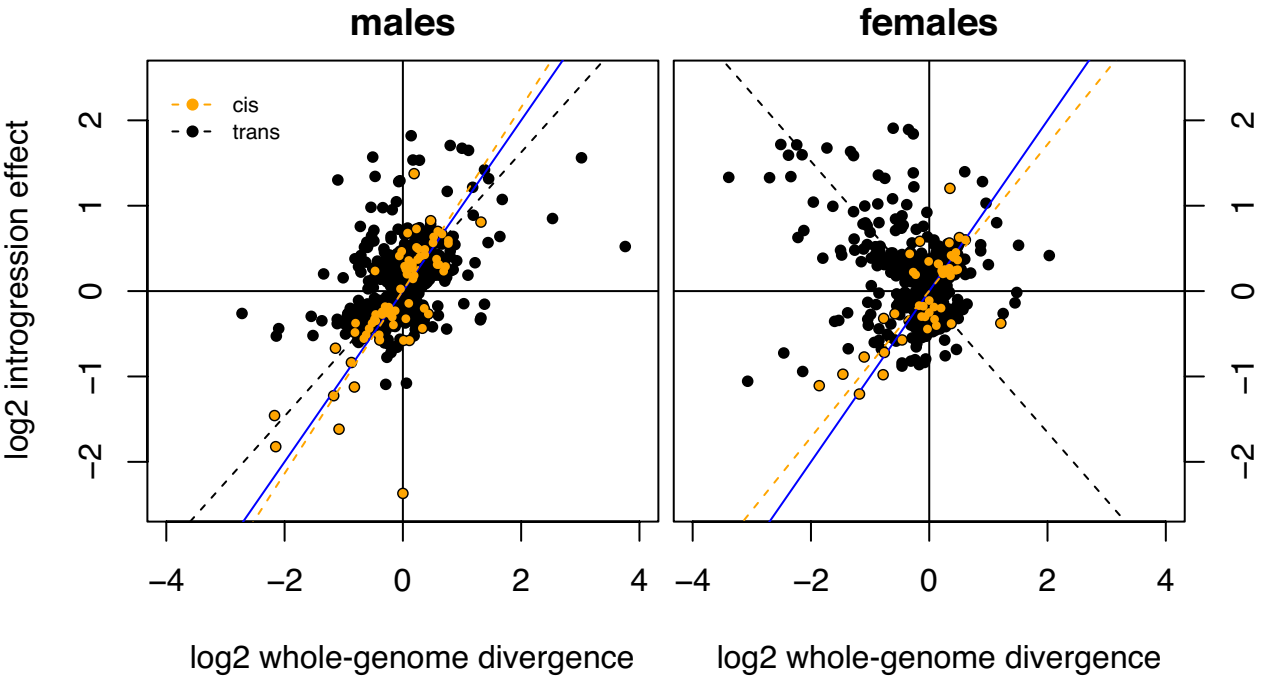

males
females
cis
trans
log2 introgression effect
log2 whole-genome divergence
log2 whole-genome divergence
−4
−2
0
2
4
−2
−1
0
1
2

Table 1. Number of detectably expressed and divergently regulated genes

| | males | | | | females | | | |
|---|---|---|---|---|---|---|---|---|
| | # genes detected | % total | # divergently regulated genes | % of divergently regulated | # genes detected | % total | # divergently regulated | % of divergently regulated |
| *genome region* | | | | | | | | |
| X | 1198 | 13.9% | 90 | 12.2% | 1552 | 16.5% | 92 | 17.2% |
| 2L | 1718 | 19.9% | 147 | 19.9% | 1689 | 18.0% | **77** | **14.4%** |
| 2R | 1865 | 21.6% | 148 | 20.1% | 1945 | 20.7% | 104 | 19.4% |
| 3L | 1733 | 20.0% | **197** | **26.7%** | 1850 | 19.7% | **143** | **26.7%** |
| 3R | 2087 | 24.1% | 154 | 20.9% | 2281 | 24.3% | 119 | 22.2% |
| 4 | 47 | 0.5% | 1 | 0.1% | 61 | 0.7% | 1 | 0.2% |
| total | 8648 | | 737 (8.5% of total) | | 9378 | | 536 (5.7% of total) | |
| introgressed region (3L) | 743 | 8.6% | **121** | **16.4%** | 776 | 8.3% | **88** | **16.4%** |
| *cis*[a] | - | - | 72 | 9.8% | - | - | 46 | 8.6% |
| *trans* | - | - | 665 | 90.2% | - | - | 490 | 91.4% |

Numbers in boldface indicate genome regions with significant deviations from expected proportions ($\chi^2$ test, $P < 0.05$)

[a]Excludes genes which failed pyrosequencing confirmation of *cis*-regulatory divergence

Table 2. *Trans*-regulatory divergence is biased towards up-regulation

| | *trans* | | *cis* | |
|---|---|---|---|---|
| | up | down | up | down |
| *males* | | | | |
| all genes | **378** | **287** | 36 | 36 |
| ≥ 1.5-fold | **40** | **7** | 9 | 8 |
| ≥ 2-fold | **18** | **2** | 1 | 6 |
| *females* | | | | |
| all genes | 249 | 241 | 23 | 23 |
| ≥ 1.5-fold | **48** | **16** | 3 | 7 |
| ≥ 2-fold | **24** | **1** | 1 | 3 |

Numbers in boldface indicate significant deviations ($P < 0.05$) from 1:1 by $\chi^2$ test

Table 3. Dominance patterns by sex, genetic control, and direction of regulation

| | | | dominance[a] | | | | *P*-value |
|---|---|---|---|---|---|---|---|
| | | | mau allele | sim allele | co-dominant | % co-dominant[b] | species bias[c] |
| *males* | *trans-regulated* | upregulated | 168 | 33 | 177 | 46.8% | **<0.0001** |
| | | downregulated | 108 | 21 | 158 | 55.1% | **<0.0001** |
| | | total | 276 | 54 | 335 | 50.4% | **<0.0001** |
| | *cis-regulated* | upregulated | 10 | 1 | 25 | 69.4% | **0.0067** |
| | | downregulated | 3 | 5 | 28 | 77.8% | 0.4795 |
| | | total | 13 | 6 | 53 | 73.6% | 0.1083 |
| *females* | *trans-regulated* | upregulated | 70 | 52 | 127 | 51.0% | 0.1032 |
| | | downregulated | 68 | 114 | 59 | 24.5% | **0.0007** |
| | | total | 138 | 166 | 186 | 38.0% | 0.1083 |
| | *cis-regulated* | upregulated | 3 | 4 | 16 | 69.6% | 0.7055 |
| | | downregulated | 3 | 6 | 14 | 60.9% | 0.3173 |
| | | total | 6 | 10 | 30 | 65.2% | 0.3173 |

[a]Dominance was assigned to the mau or sim allele if the 95% confidence intervals around the *d* estimate did not overlap 0; confidence intervals overlapping 0 were classified as co-dominant

[b]The proportion of co-dominant alleles is significantly greater for expression traits regulated in *cis* than in *trans* in both sexes ($\chi^2$ test, $P < 0.0005$)

[c]*P*-value species bias was determined by a $\chi^2$ test comparing the proportion of *mau* and *sim* dominant alleles with a 1:1 expectation

Table 4. Gene misexpression results more frequently from divergent *trans*-regulation than *cis*

| | *trans* | | *cis* | |
|---|---|---|---|---|
| | misexpressed | not | misexpressed | not |
| *males* | | | | |
| all genes | 394 | 271 | 37 | 35 |
| $\geq$ 1.5-fold | 38 | 9 | 9 | 8 |
| $\geq$ 2-fold | 15 | 5 | 4 | 3 |
| *females* | | | | |
| all genes | **362** | **128** | **24** | **22** |
| $\geq$ 1.5-fold | **55** | **9** | **3** | **7** |
| $\geq$ 2-fold | **23** | **2** | **1** | **3** |

Rows in boldface indicate the proportion of misexpressed genes is significantly different (Fisher's Exact Test $P \leq 0.01$) between *trans*- and *cis*-regulated genes

## Supplemental methods

### Microarray hybridization and normalization

Microarrays were constructed from 21,504 exon-specific PCR products amplified from the Oregon-R strain of *D. melanogaster* (Hild et al. 2003). PCR products were spotted onto poly-L-lysine coated slides (Thermo Scientific, Portsmouth, NH) following standard protocols (www.microarray.org). Competitive hybridizations followed a modified loop design (Supplementary Figure 7) that minimizes the distance between any two genotypes (Townsend 2003). Each genotype was assayed on four replicate arrays and separate loops were used for males and females.

Sequence mismatches resulting from divergence between species influences hybridization efficiency on microarrays and can confound inferences of differential gene expression (Gilad et al. 2005). However, in all the genotypes used here except for *mau12*, genes located outside the introgressed segment (>90% of the genome) are derived from the *sim*B strain. Interspecific divergence therefore will not influence estimates of changes in expression at genes located outside the introgressed segments (*i.e. trans*-regulated genes). *D. simulans* and *D. mauritiana* are equally divergent from *D. melanogaster* (Hey and Kliman 1993; Kliman and Hey 1993; Garrigan et al. 2012), and so *D. simulans* and *D. mauritiana* messages transcribed from genes within the introgressed segments will be equally divergent, on average, from the *D. melanogaster* sequences spotted on the microarray. We rule out spurious inference of *cis*-regulatory effects resulting from gene-specific and lineage-specific sequence divergence by means of pyrosequencing assays of allele-specific expression in introgression heterozygotes.

RNA was extracted from frozen flies using TRIzol (Invitrogen, Carlsbad, CA), chloroform and phase-lock gel tubes (5 Prime, Gaithersburg, MD) to separate aqueous and organic layers. RNA was independently extracted from multiple pools of flies from all three cohorts and pooled for cDNA synthesis. At least 20μg total RNA was used as a template for cDNA synthesis (SuperScript II, Invitrogen, Carlsbad, CA), and incorporation of fluorescent dyes and hybridization to microarrays were done with the 3DNA Array50 kit (Genisphere, Hatfield, PA) following the manufacturer's protocols. Slides were scanned in an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA) and microarray images were processed with GenePix v5.0 (Molecular Devices, Sunnyvale, CA).

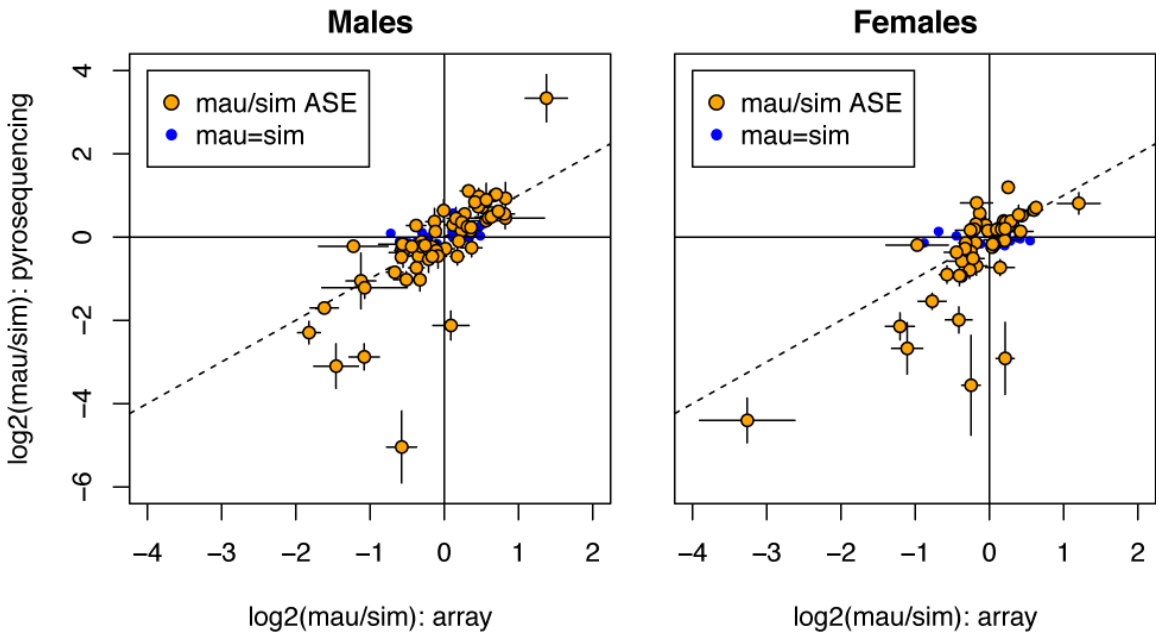
Microarray spots were filtered using the following quality control criteria: 70% of the foreground pixels within a spot were required to have a signal intensity higher than two standard deviations above the median background signal intensity in at least one of the two channels (Cy3 or Cy5); the median foreground signal intensity was required to be at least three times as great as the median background signal intensity in at least one of the two channels; a spot must have more than 30 foreground pixels. Spot-wise log2 ratios were normalized over overall spot signal intensity by loess smoothing (Smyth 2004) using a span of 0.3. To account for spatial variation in signal across arrays, spot-wise

log2 ratios were subsequently normalized by a second loess smoothing over the physical location of the spots on the slide, using a span of 0.002, which corresponds to an effective neighborhood of ~ 250 spots. Only probes that could be computationally localized to a single position on one of the six chromosome arms in the assembled *D. melanogaster* genome (R5.48) were retained for analysis.

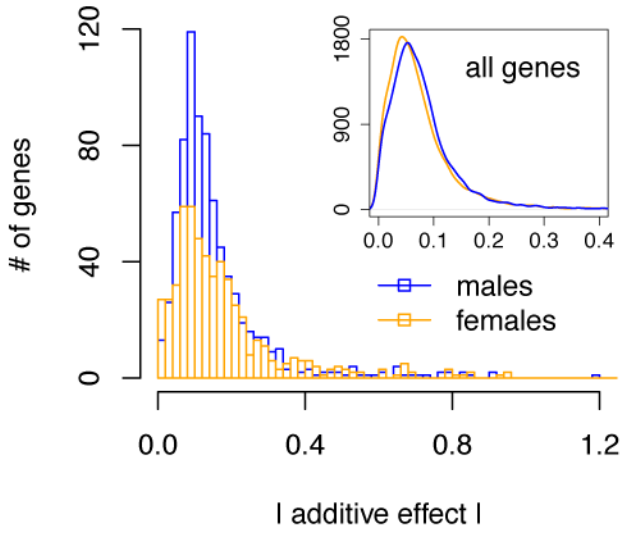
**Pyrosequencing**

Allele-specific expression was assayed by pyrosequencing 90 candidate genes identified from the microarrays as potentially harboring *cis*-regulatory variants (Supplementary Figure 8, Supplementary Table 8). Candidates included genes with significant evidence for eQTL in one or both sexes, as well as candidates with associations that were significant with a less stringent cutoff than was used for the final analysis. Each gene was assayed in males and females separately using two replicate RNA extractions from two different heterozygous introgression genotypes generated from both reciprocal crosses, resulting in eight biologically independent replicates in each sex. RNA was extracted using the same TRIzol/chloroform/phase-lock gel protocol as for the microarrays, and at least 2.5μg RNA was DNase treated (NEB, Ipswitch, MA) and used as a template for cDNA synthesis (SuperScript III, Invitrogen, Carlsbad, CA) using oligo-dT priming. Genomic DNA was extracted from heterozygous introgression females using phenol, chloroform, phase-lock gel tubes and an RNaseA treatment.

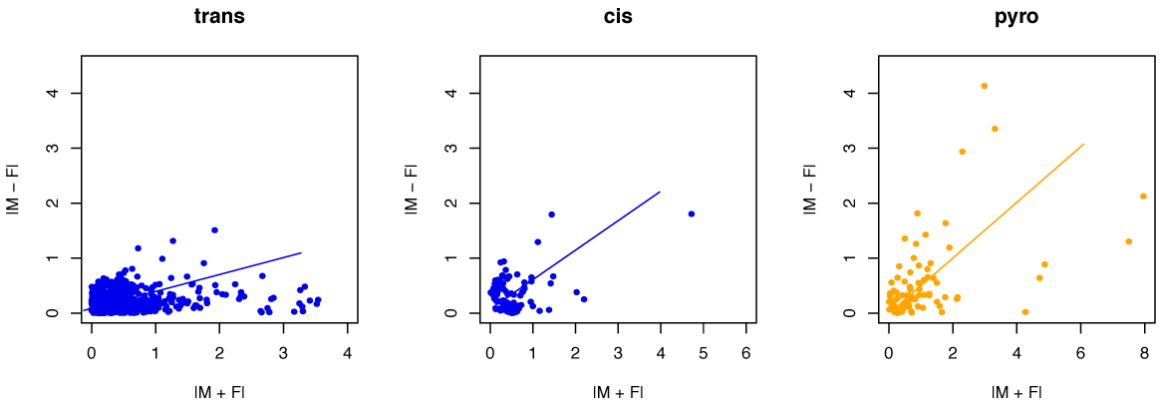
Each transcript to be assayed was PCR amplified (Promega GoTaq, Madison, WI) from cDNA and the PCR products were used in pyrosequencing reactions (Qiagen, Valencia, CA) following standard protocols (Wittkopp 2011). Two independent genomic DNA samples for each heterozygous introgression genotype were assayed by pyrosequencing and the resulting ratios were used to normalize the corresponding measurements of allele-specific expression from cDNA. Pyrosequencing ratios from gDNA at four genes differed strongly between the two heterozygous introgression genotypes, and Sanger sequencing identified SNPs resulting from *de novo* mutation or gene conversion in the sequences targeted by the pyrosequencing primers in the genotypes with unexpected ratios. These genotypes were removed from the analysis, and these four genes were analyzed using only one heterozygous introgression genotype (Supplementary Table 8).



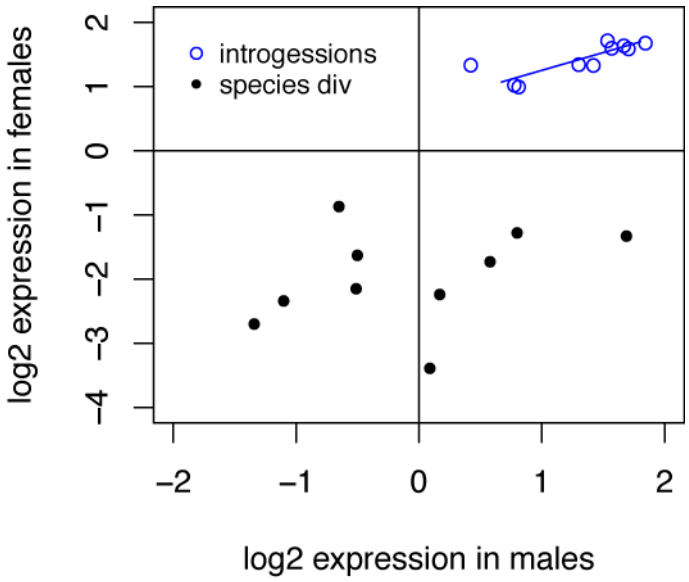

**Supplemental Figure 1.** Confirming candidate *cis*-regulatory variants with measures of allele-specific expression from pyrosequencing. Plotted on the ordinate is the homozygous introgression expression effect relative to the background *D. simulans* strain; on the abscissa is the log2 ratio of expression from the *D. mauritiana* and *D. simulans* alleles in introgression heterozygotes. The dashed line has a slope of 1. Error bars are only shown for genes with significant allelic differences in expression (ASE) and indicate 95% confidence limits for expression estimates. If differences in expression were due entirely to *cis*-regulatory divergence, then the ratio of allelic expression in an introgression heterozygote should equal the ratio of expression between introgression homozygotes and *sim*B. Deviations from this expectation can result from *cis*-by-*trans* genetic interactions (Wittkopp et al. 2004), as well as from comparing expression estimates using microarrays and pyrosequencing in these experiments. The allele-specific ratios obtained from pyrosequencing and the effects estimated from the microarrays are generally in good agreement ($\rho = 0.744$ and 0.719 in males and females, respectively; $P < 0.0001$), suggesting minimal *cis*-by-*trans* interaction effects. This result is expected, as > 90% of *trans*-acting factors in introgression homozygotes, introgression heterozygotes, and *sim*B are derived from the same inbred *D. simulans* strain.



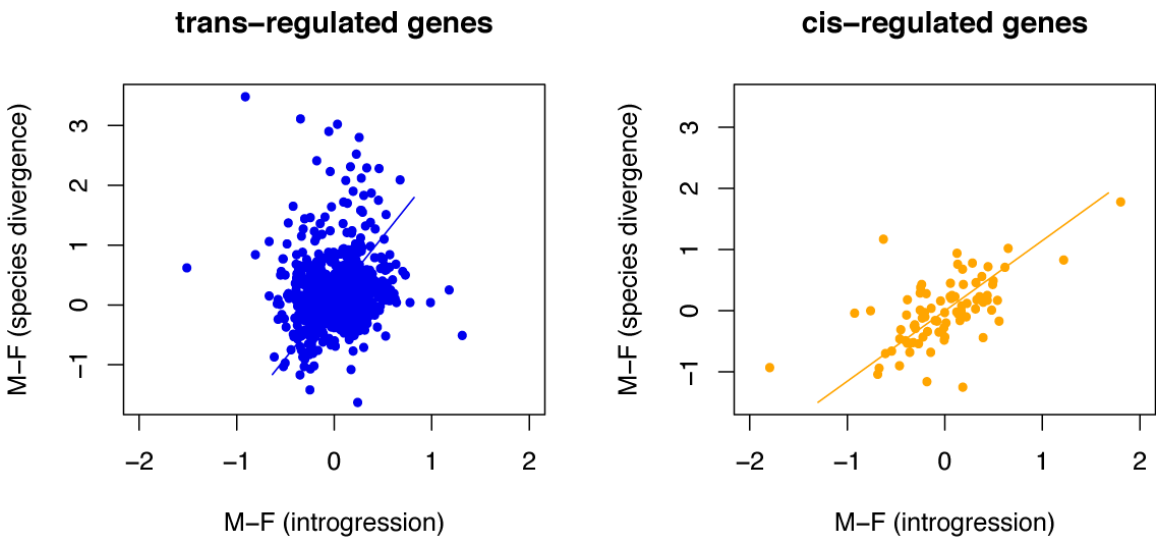

**Supplemental Figure 2**. Distributions of the absolute magnitude of additive effects of introgressed factors on gene expression in males and females. Divergently regulated genes are shown in the main panel; inset shows distributions for additive effects associated with the best fit marker for all genes considered in the eQTL analysis, regardless of the statistical significance of association with a genetic marker.



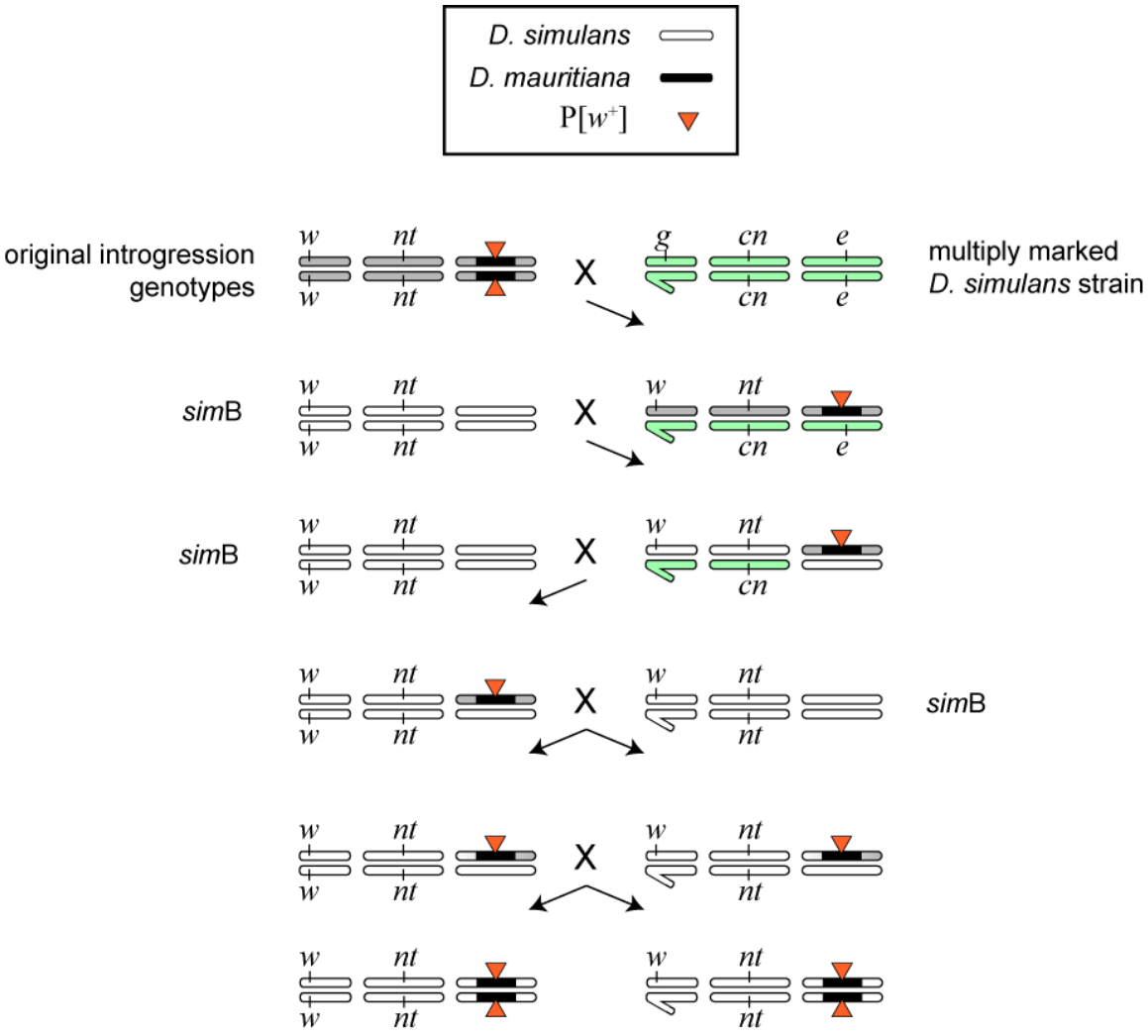

**Supplemental Figure 3.** Greater sexual dimorphism in expression is associated with larger average effects among introgressed *trans*-regulatory factors (slope = 0.31, $P < 0.0001$) as well as introgressed *cis*-regulatory factors assayed both by microarray (slope = 0.53, $P < 0.0001$) and pyrosequencing (slope = 0.51, $P < 0.0001$).



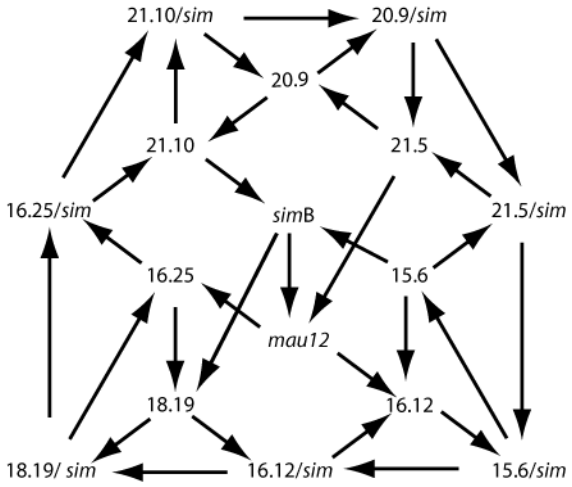

**Supplemental Figure 4.** Homozygous introgression effects on the expression of ten divergently regulated immunity-related genes are positively correlated between males and females ($\rho = 0.78$, $P = 0.008$), but whole-genome divergence effects on the expression of these 10 genes are uncorrelated between the sexes ($P = 0.32$).



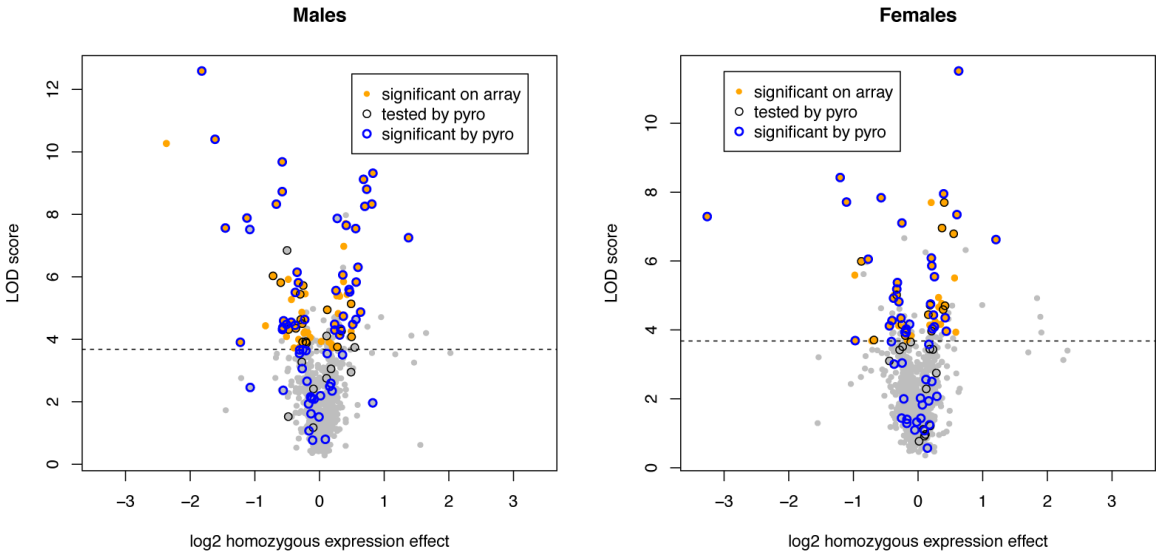

**Supplemental Figure 5.** Sexually dimorphic expression resulting from introgressed *trans*-regulatory factors is poorly correlated with sexually dimorphic expression at the same genes due to whole-genome divergence between *sim*B and *mau12* (slope = 2.04, $\rho$ = 0.124, $P$ = 0.0002) compared to sexually dimorphic expression resulting from introgressed *cis*-regulatory sequences (slope = 1.15, $\rho$ = 0.607, $P$ < 0.0001).



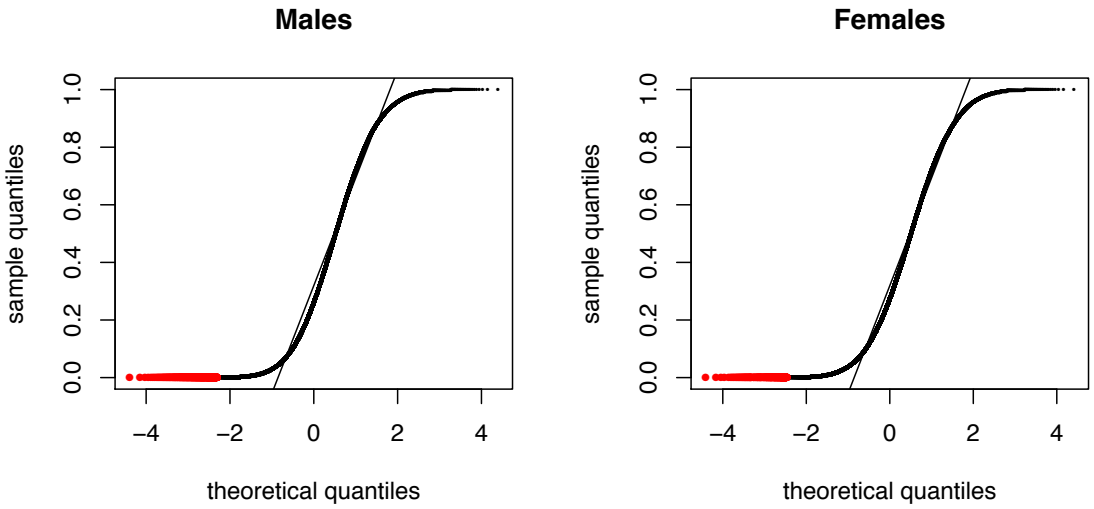

**Supplemental Figure 6.** Crossing scheme used to re-extract introgression 3rd chromosomes into the *sim*B genetic background (which is the same strain used for the original introgressions). Shown are the sex chromosomes and two major autosomes and the visible mutations they carry. Passage of introgression chromosomes through females in the 4th and 5th generations allows recombination and replaced some regions of chromosome arm 3L outside of the introgressed segments with genetic material from *sim*B.




**Supplemental Figure 7.** Loop design used for comparative hybridizations. Arrowhead indicates the sample labeled with Cy5; alternate sample was labeled with Cy3.




**Supplemental Figure 8.** Volcano plots indicate genes harboring candidate *cis*-regulatory divergence chosen for pyrosequencing analysis. Grey dots indicate all genes detected as expressed and located inside the introgressed region of the genome. The dashed line indicates the threshold LOD score chosen to determine significant associations between expression phenotypes and marker genotypes. Genes marked by grey dots above this threshold had expression phenotypes associated with markers distant from the gene.




**Supplemental Figure 9.** Q-Q plots of QTL mapping *P*-values. Lines correspond to a normal distribution with the same first and third quartiles as the data. Points in red indicate *P*-values retained after FDR correction for multiple tests.

**Supplemental Table 1.** Pyrosequencing confirmation of candidate *cis*-regulatory variants

| | **males** | **females** |
|---|---|---|
| *cis*-candidates identified by microarray | 86 | 60 |
| candidates assayed by pyrosequencing | 49 | 41 |
| significant allele-specific expression | 37<br>75.5% | 30<br>73.2% |
| significant ASE confirms array result | 35<br>71.4% | 27<br>65.9% |
| all genes assayed by pyrosequencing | 90 | 85 |
| significant allele-specific expression | 68<br>75.6% | 60<br>70.6% |
| *significant ASE* | | |
| sim allele > mau allele | 37 | 34 |
| mau allele > sim allele | 31 | 26 |
| *allelic ratio > 2-fold* | | |
| sim allele > mau allele | 11 | 10 |
| mau allele > sim allele | 4 | 2 |

**Supplemental Table 2**. More genes are divergently regulated in males than in females

| | **all genes** | | ***cis*** | | ***trans*** | |
|---|---|---|---|---|---|---|
| | **males** | **females** | **males** | **females** | **males** | **females** |
| *all genes detectably expressed* | 8648 | 9378 | 743 | 776 | 8398 | 9143 |
| FDR | | | | | | |
| 0.05 | **2039** | **1719** | 108 | 84 | **1931** | **1635** |
| 0.04 | **1642** | **1337** | 96 | 77 | **1546** | **1260** |
| 0.03 | **1215** | **939** | 85 | 64 | **1130** | **875** |
| 0.025 | **964** | **733** | 79 | 56 | **885** | **677** |
| 0.02 | **737** | **536** | **72** | **46** | **665** | **490** |
| 0.01 | **340** | **229** | **48** | **26** | **292** | **203** |
| 0.005 | **164** | **97** | **36** | **18** | **128** | **79** |
| 0.001 | 40 | 31 | 20 | 10 | 20 | 21 |

Numbers in boldface indicate the numbers of divergently regulated genes in males and females is significantly different from expected ($\chi^2$ test, $P < 0.01$).

**Supplemental Table 3.** Germline-specific genes are not overrepresented among divergently regulated genes

| | **Males** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | all genes detected on the array | | | | genes with significant linkage | | | |
| tissue with maximal expression | | tissue-specific genes | | | | tissue-specific genes | | |
| | | $\tau > 0.85$ | $\tau > 0.90$ | $\tau > 0.95$ | | $\tau > 0.85$ | $\tau > 0.90$ | $\tau > 0.95$ |
| accessory gland | 291 | 78 | 69 | 40 | 30 | 6 | 5 | 4 |
| adult abdominal-thoracic ganglion | 267 | 26 | 8 | 5 | 18 | 2 | 0 | 0 |
| adult brain | 381 | 76 | 36 | 8 | 34 | 9 | 4 | 0 |
| adult crop | 242 | 9 | 5 | 2 | 27 | 1 | 0 | 0 |
| adult eye | 296 | 53 | 43 | 25 | 23 | 5 | 5 | 3 |
| adult fatbody | 188 | 8 | 5 | 5 | 15 | 1 | 1 | 1 |
| adult heart | 128 | 15 | 12 | 5 | 14 | 2 | 2 | 1 |
| adult hindgut | 114 | 16 | 8 | 4 | 13 | 4 | 3 | 2 |
| adult midgut | 192 | 58 | 40 | 11 | 35 | 8 | 5 | 1 |
| adult salivary gland | 353 | 11 | 3 | 0 | 39 | 1 | 1 | 0 |
| ejaculatory duct | 159 | 18 | 8 | 5 | 13 | 1 | 1 | 0 |
| female spermatheceae (virgin) | 230 | 6 | 4 | 2 | 32 | 2 | 2 | 1 |
| larval CNS | 382 | 26 | 17 | 7 | 39 | 1 | 1 | 0 |
| larval hindgut | 83 | 13 | 7 | 1 | 9 | 0 | 0 | 0 |
| larval malpighian tubules | 354 | 29 | 23 | 12 | 39 | 2 | 2 | 1 |
| larval midgut | 145 | 40 | 25 | 5 | 26 | 5 | 3 | 0 |
| larval salivary gland | 338 | 7 | 7 | 5 | 21 | 0 | 0 | 0 |
| larval trachea | 215 | 29 | 20 | 13 | 22 | 3 | 1 | 1 |
| ovary | 912 | 34 | 19 | 9 | 78 | 7 | 7 | 4 |
| testis | 1184 | 849 | 786 | 675 | 131 | 100 | 91 | 80 |
| total | 6454 | 1401 | 1145 | 839 | 658 | 160 | 134 | 99 |
| % germline specific | | 13.2% | 12.2% | 10.5% | | 15.2% | 13.8% | 12.2% |

**Supplemental Table 3 (continued).** Germline-specific genes are not overrepresented among divergently regulated genes

| | Females | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | all genes detected on the array | | | | genes with significant linkage | | | |
| tissue with maximal expression | | tissue-specific genes | | | | tissue-specific genes | | |
| | | $\tau > 0.85$ | $\tau > 0.90$ | $\tau > 0.95$ | | $\tau > 0.85$ | $\tau > 0.90$ | $\tau > 0.95$ |
| accessory gland | 264 | 23 | 18 | 13 | 14 | 1 | 1 | 1 |
| adult abdominal-thoracic ganglion | 320 | 30 | 11 | 7 | 21 | 4 | 1 | 0 |
| adult brain | 491 | 99 | 53 | 16 | 30 | 10 | 4 | 2 |
| adult crop | 287 | 18 | 11 | 3 | 10 | 2 | 1 | 0 |
| adult eye | 320 | 60 | 47 | 29 | 20 | 5 | 4 | 3 |
| adult fatbody | 214 | 11 | 6 | 5 | 13 | 0 | 0 | 0 |
| adult heart | 164 | 21 | 17 | 9 | 16 | 3 | 2 | 1 |
| adult hindgut | 136 | 20 | 12 | 7 | 6 | 3 | 1 | 0 |
| adult midgut | 244 | 73 | 54 | 14 | 58 | 16 | 10 | 2 |
| adult salivary gland | 381 | 16 | 7 | 3 | 25 | 2 | 0 | 0 |
| ejaculatory duct | 163 | 13 | 7 | 3 | 16 | 0 | 0 | 0 |
| female spermatheceae (virgin) | 267 | 13 | 10 | 6 | 16 | 2 | 2 | 1 |
| larval CNS | 527 | 39 | 27 | 11 | 22 | 3 | 1 | 0 |
| larval hindgut | 104 | 26 | 14 | 6 | 10 | 0 | 0 | 0 |
| larval malpighian tubules | 412 | 35 | 29 | 15 | 20 | 3 | 2 | 1 |
| larval midgut | 188 | 57 | 39 | 11 | 24 | 12 | 6 | 2 |
| larval salivary gland | 395 | 11 | 11 | 7 | 24 | 2 | 2 | 1 |
| larval trachea | 259 | 40 | 29 | 17 | 19 | 2 | 2 | 1 |
| ovary | 1493 | 96 | 71 | 40 | 70 | 8 | 5 | 4 |
| testis | 521 | 183 | 159 | 122 | 41 | 18 | 14 | 9 |
| total | 7150 | 884 | 632 | 344 | 475 | 96 | 58 | 28 |
| % germline specific | | 1.3% | 1.0% | 0.6% | | 1.7% | 1.1% | 0.8% |

**Supplemental Table 4**. Sexual dimorphism in divergent regulation

| | **males** | **females** |
|---|---|---|
| *genes with expression phenotypes significantly associated with a genetic marker* | 737 | 536 |
| *genes with significant association in at least one sex and expression detected in both sexes* | 613<br>83.2% | 416<br>77.6% |
| *detected in both sexes but significant in one sex only* | 528<br>86.1% | 331<br>79.6% |
| *detected in both and significantly associated in both sexes* | 86<br>11.67% | 86<br>16.04% |
| *associated with the same or neighboring markers* | 67<br>9.1% | 67<br>12.5% |
| *associated with distant markers* | 23<br>3.12% | 23<br>4.29% |

**Supplemental Table 5**. *cis*-divergence effects are larger than *trans*-divergence effects

| | median \| 2a \|[a] | | Mann-Whitney |
|---|---|---|---|
| | *cis* | *trans* | *P*-value |
| males | 0.386 | 0.228 | < 0.0001 |
| females | 0.339 | 0.250 | 0.0007 |

[a]the absolute value of twice the additive effect estimated from the QTL model

**Supplemental Table 6.** Narrowly expressed genes show larger introgression effects, greater interspecific divergence, and more sexual dimorphism in expression than broadly expressed genes

| comparison | divergent regulation | median log2 fold-difference in expression | | | | *P*-value (M-W test) | |
|---|---|---|---|---|---|---|---|
| | | broad[a] $\tau < 0.4$ | intermediate $0.4 \leq \tau \leq 0.9$ | tissue-specific $\tau > 0.9$ | no data | specific/ broad | broad vs. tissue-specific |
| \|M-F\| introgression effect | all | 0.169 | 0.200 | 0.217 | 0.250 | **1.3** | **<0.0001** |
| \|M-F\| introgression effect | *trans* | 0.164 | 0.196 | 0.211 | 0.246 | **1.3** | **0.0002** |
| \|M-F\| introgression effect | *cis* | 0.222 | 0.288 | 0.518 | 0.358 | **2.3** | **0.0058** |
| \|M-F\| species divergence | all | 0.190 | 0.310 | 0.300 | 0.310 | **1.6** | **0.0001** |
| \|M-F\| species divergence | *trans* | 0.190 | 0.280 | 0.280 | 0.310 | **1.5** | **0.0007** |
| \|M-F\| species divergence | *cis* | 0.250 | 0.440 | 0.610 | 0.440 | 2.4 | 0.0660 |
| \|M\| introgression effect | all | 0.096 | 0.149 | 0.137 | 0.157 | **1.4** | **<0.0001** |
| \|M\| introgression effect | *trans* | 0.093 | 0.145 | 0.129 | 0.144 | **1.4** | **<0.0001** |
| \|M\| introgression effect | *cis* | 0.148 | 0.237 | 0.278 | 0.227 | **1.9** | **0.0003** |
| \|M\| species divergence | all | 0.160 | 0.230 | 0.315 | 0.230 | **2.0** | **<0.0001** |
| \|M\| species divergence | *trans* | 0.150 | 0.230 | 0.300 | 0.220 | **2.0** | **<0.0001** |
| \|M\| species divergence | *cis* | 0.290 | 0.540 | 0.370 | 0.395 | 1.3 | 0.3150 |
| \|F\| introgression effect | all | 0.102 | 0.154 | 0.159 | 0.175 | **1.6** | **<0.0001** |
| \|F\| introgression effect | *trans* | 0.095 | 0.149 | 0.151 | 0.175 | **1.6** | **<0.0001** |
| \|F\| introgression effect | *cis* | 0.151 | 0.222 | 0.240 | 0.190 | 1.6 | 0.3098 |
| \|F\| species divergence | all | 0.140 | 0.260 | 0.240 | 0.210 | **1.7** | **0.0023** |
| \|F\| species divergence | *trans* | 0.130 | 0.240 | 0.240 | 0.210 | **1.8** | **0.0045** |
| \|F\| species divergence | *cis* | 0.270 | 0.455 | 0.665 | 0.370 | 2.5 | 0.2419 |
| \|M-F\| allelic expression | allele-specific ratio | 0.241 | 0.273 | 0.639 | 0.438 | **2.7** | **0.0030** |
| \|M\| allelic expression | allele-specific ratio | 0.239 | 0.340 | 0.562 | 0.462 | **2.4** | **0.0088** |
| \|F\| allelic expression | allele-specific ratio | 0.193 | 0.433 | 0.428 | 0.734 | **2.2** | **0.0301** |

[a]tissue breadth in expression was determined from data obtained from *D. melanogaster* (FlyAtlas); see Methods

*P*-values in boldface are significant at FDR = 0.05 (table-wise).

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