

Transcriptome Differences between Alternative Sex Determining Genotypes in the House Fly, *Musca domestica*

Richard P. Meisel^{1,*}, Jeffrey G. Scott², and Andrew G. Clark³

¹Department of Biology and Biochemistry, University of Houston

²Department of Entomology, Cornell University

³Department of Molecular Biology and Genetics, Cornell University

*Corresponding author: E-mail: rpmeisel@uh.edu.

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Abstract

Sex determination evolves rapidly, often because of turnover of the genes at the top of the pathway. The house fly, *Musca domestica*, has a multifactorial sex determination system, allowing us to identify the selective forces responsible for the evolutionary turnover of sex determination in action. There is a male determining factor, *M*, on the *Y* chromosome (Y^M), which is probably the ancestral state. An *M* factor on the third chromosome (III^M) has reached high frequencies in multiple populations across the world, but the evolutionary forces responsible for the invasion of III^M are not resolved. To test whether the III^M chromosome invaded because of sex-specific selection pressures, we used mRNA sequencing to determine whether isogenic males that differ only in the presence of the Y^M or III^M chromosome have different gene expression profiles. We find that more genes are differentially expressed between Y^M and III^M males in testis than head, and that genes with male-biased expression are most likely to be differentially expressed between Y^M and III^M males. We additionally find that III^M males have a “masculinized” gene expression profile, suggesting that the III^M chromosome has accumulated an excess of male-beneficial alleles because of its male-limited transmission. These results are consistent with the hypothesis that sex-specific selection acts on alleles linked to the male-determining locus driving evolutionary turnover in the sex determination pathway.

Key words: sex determination, gene expression, RNA-Seq, sex-specific selection, sex chromosomes.

Introduction

Sex determination (SD) is an essential developmental process responsible for sexually dimorphic phenotypes. It is therefore paradoxical that SD pathways are poorly conserved, with master SD (MSD) genes at the top of the pathway differing between closely related species and even variable within species (Bull 1983; Wilkins 1995; Pomiankowski et al. 2004; Beukeboom and Perrin 2014). The hypotheses to explain the rapid evolution of SD pathways fall into three categories. First, SD evolution may be selectively neutral if MSD turnover is the result of mutational input without phenotypic or fitness consequences (van Doorn 2014). Second, frequency dependent (sex-ratio) selection could favor a new MSD variant if one sex is below its equilibrium frequency (Eshel 1975; Bull and Charnov 1977; Bulmer and Bull 1982; Werren and Beukeboom 1998). Third, a new MSD locus can invade a population if the new MSD variant itself or genetically linked alleles confer a fitness

benefit (Charlesworth D and Charlesworth B 1980; Rice 1986; Charlesworth 1991; van Doorn and Kirkpatrick 2007, 2010). Those fitness effects could be beneficial to both sexes (natural selection), increase the reproductive success of the sex determined by the new MSD variant (sexual selection), or be beneficial to the sex determined by the MSD variant and deleterious to the other sex (sexually antagonistic selection). Sexually antagonistic selection is predicted to be an especially important driver of MSD turnover because linkage to an MSD locus allows the sexually antagonistic allele to be inherited in a sex-limited manner, thereby resolving the intersexual conflict (Charlesworth D and Charlesworth B 1980; van Doorn and Kirkpatrick 2007, 2010; Roberts et al. 2009).

The house fly, *Musca domestica*, is an ideal model for testing hypotheses about the evolution of SD because it has a multifactorial SD system, with male- and female-determining loci

segregating in natural populations (Dübendorfer et al. 2002; Hamm et al. 2015). Most relevant to the work presented here is the fact that the male-determining factor, M , can be located on the Y chromosome (Y^M), any of the five autosomes (A^M), and even the X chromosome (Hamm et al. 2015). It is unknown whether these M -factors are the same gene in different locations or different genes that have independently assumed the role of an MSD locus (Bopp 2010). Y^M is a common arrangement (Hamm et al. 2015), and it is thought to be the ancestral state because it is the genotype found in close relatives of the house fly (Boyes et al. 1964; Boyes and Van Brink 1965; Dübendorfer et al. 2002). M on the third chromosome (III^M) is also common, but it is not clear what was responsible for the invasion of the III^M chromosome (Hamm et al. 2015). Note that when the M factor arrived on chromosome III , this entire chromosome essentially assumed Y-like properties, including male-biased transmission and reduced recombination (Hamm et al. 2015). However, the III^M chromosome is not a degenerated Y chromosome because III^M homozygotes are viable, fertile, and found in natural populations (Hamm et al. 2015). Identifying the selective forces responsible for the invasion of III^M will be a powerful test of the hypotheses to explain SD evolution.

Strong linkage to A^M is expected for alleles on the same autosome because recombination is low or nonexistent in house fly males (Hiroyoshi 1961; Hamm et al. 2015), but see Feldmeyer et al. (2010). It is possible that A^M chromosomes invaded house fly populations because of selection on phenotypic effects of either the autosomal M loci themselves or alleles linked to M -factors (Franco et al. 1982; Tomita and Wada 1989a; Kozielska et al. 2006; Feldmeyer et al. 2008). M variants are known to have subtle phenotypic effects, which include differential splicing and expression of SD pathway genes between Y^M and A^M males (Schmidt et al. 1997; Hediger et al. 2004; Siegenthaler et al. 2009). In addition, A^M chromosomes form stable latitudinal clines on multiple continents (Franco et al. 1982; Tomita and Wada 1989b; Hamm et al. 2005; Kozielska et al. 2008), and seasonality in temperature is somewhat predictive of their distribution (Feldmeyer et al. 2008). Furthermore, in laboratory experiments, III^M males outcompeted Y^M males for female mates; the III^M chromosome increased in frequency over generations in population cages; and III^M males had higher rates of emergence from pupae than Y^M males (Hamm et al. 2009). The most specific phenotype that has been linked to A^M is insecticide resistance (Kerr 1960, 1961, 1970; Denholm et al. 1983; Kence M and Kence A 1992), but insecticide resistance alone cannot entirely explain the invasion of A^M chromosomes (Shono and Scott 1990; Hamm et al. 2005). These results all support the hypothesis that natural, sexual, or sexually antagonistic selection on M variants or linked alleles drove the invasion of A^M chromosomes.

To test whether sex-specific selection pressures could be responsible for the invasion of the III^M chromosome, we

used high-throughput mRNA sequencing (mRNA-Seq) to compare gene expression profiles between nearly isogenic Y^M and III^M males that only differ in their M -bearing chromosome. These contrasts are essentially a comparison between flies with the ancestral Y chromosome (Y^M) and individuals with a recently evolved “neo-Y” (III^M). The gene expression differences we detected were the result of both differentiation of *cis* regulatory regions between the III^M and “standard” third chromosome and *trans* effects of the III^M and/or Y^M chromosome(s) on expression throughout the genome. We found that genes responsible for male phenotypes are more likely to be differentially expressed between Y^M and III^M males, suggesting that Y^M and III^M males have phenotypic differences that would be differentially affected by male-specific selection pressures. We also found that III^M males have a “masculinized” gene expression profile. These results support the hypothesis that sexual or sexually antagonistic selection drives evolutionary turnover at the top of SD pathways.

Materials and Methods

Strains

We compared gene expression between two nearly isogenic house fly strains that differ only in the chromosome carrying M . The first, Cornell susceptible (CS), is an inbred, lab adapted strain with XX males that are heterozygous for a III^M chromosome and a standard third chromosome that lacks an M factor (XX ; III^M/III^{CS}) (Scott et al. 1996; Hamm et al. 2005) (fig. 1A). CS females are XX and homozygous for the standard third chromosome (XX ; III^{CS}/III^{CS}). We created a strain with Y^M males that has the X chromosome and all standard autosomes from the CS strain. To do so, we used a backcrossing approach to move the Y chromosome from the genome strain (aabys) onto the CS background (fig. 1B), creating the strain CS-aabys-Y (CSaY). CSaY males are XY and homozygous for the standard CS third chromosome (XY ; III^{CS}/III^{CS}). The aabys strain has a recessive phenotypic marker on each of the five autosomes (Wagoner 1967; Tomita and Wada 1989b). To confirm that the aabys autosomes had been purged from the CSaY genome, we crossed CSaY flies to aabys and observed only wild-type progeny. CS and CSaY males are nearly isogenic, differing only in that CS males are XX and heterozygous for the III^M and standard III^{CS} chromosomes, and CSaY males are XY and homozygous for the standard III^{CS} chromosome (fig. 1). Females are genetically identical between strains.

We are confident that the strains are isogenic except for the M -bearing chromosome because there is very little evidence for recombination in male house flies with an XY genotype (Hiroyoshi 1961; Hamm et al. 2015). However, if there were minimal recombination between the CS and aabys chromosomes in our crossing scheme, the majority of autosomal alleles in the CSaY strain would still have originated from the CS genotype, with very little contribution from aabys autosomes.

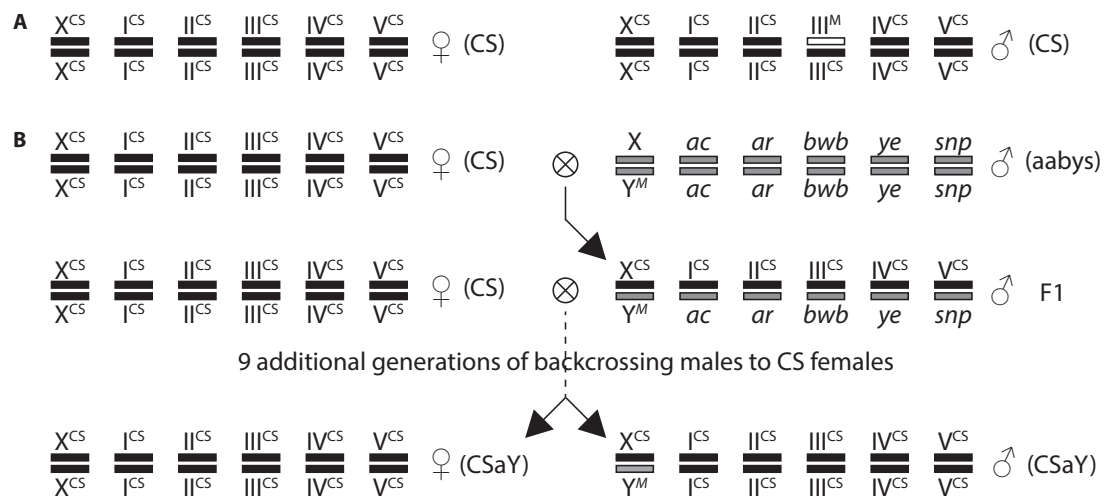


Fig. 1.—Genotypes of flies. (A) Genotypes of CS males and females. (B) Crossing scheme used to generate the CSaY (Y^M) strain. Each pair of parallel rectangles represents homologous chromosomes; there is one pair of sex chromosomes (X and Y) and five autosomes. Chromosomes of CS origin are black and indicated by “CS,” except for the III^M chromosome which is white. Chromosomes of aabys origin are gray, and the aabys strain has a recessive phenotypic marker on each autosome. Females from the CS strain were crossed to aabys males, and the male progeny were backcrossed to CS females for ten generations to create the CSaY strain. Because there is no recombination in XY males, CS and CSaY are isogenic except that the CSaY males have a Y chromosome and CS males have a III^M chromosome.

Samples and mRNA-Seq

CS and CSaY flies were kept at 25 °C with a 12:12 h light:dark cycle. Larvae were reared in media made with 1.8l water, 500g calf manna (Manna Pro, St. Louis, MO), 120g bird and reptile litter wood chips (Northeastern Products, Warrensburg, NY), 60g dry active baker’s yeast (MP Biomedical Solon, OH), and 1.21kg wheat bran (Cargill Animal Nutrition, Minneapolis, MN), as described previously (Hamm et al. 2009).

We sampled two types of tissue from CS and CSaY males and females: Head and gonad. All dissections were performed on living, nonanesthetized 4- to 6-day-old unmated adult flies. Heads were separated from males and females, homogenized in TRIzol reagent (Life Technologies) using a motorized grinder, and RNA was extracted on QIAGEN RNeasy columns following the manufacturer’s instructions including a genomic DNA (gDNA) elimination step. Testes were dissected from males, and ovaries were dissected from females in Ringer’s solution (182 mM KCl, 46 mM NaCl, 3 mM CaCl₂, 10 mM Tris-Cl in ddH₂O). Ovary and testis samples were dissolved in TRIzol and RNA was extracted on QIAGEN RNeasy columns with gDNA elimination. Three biological replicates of CS (III^M) male heads, CSaY (Y^M) male heads, CS testes, and CSaY testes were collected; one sample was collected for each of the four female dissections (CS head, CSaY head, CS ovary, and CSaY ovary).

Barcoded mRNA-Seq libraries were prepared using the Illumina TruSeq kit following the manufacturer’s instructions. Briefly, mRNA was purified using oligo-dT magnetic beads,

cDNA was synthesized using random hexamer primers, and sequencing libraries were constructed using the cDNA. The samples were run on two lanes of an Illumina HiSeq2500 at the Cornell Medical School Genomics Resources Core Facility. One lane had the eight head samples, and the other lane had the eight gonad (testis and ovary) samples. We generated 101-bp single-end reads, and the sequencing reads were processed using Casava 1.8.2.

mRNA-Seq Data Analysis

Illumina mRNA-Seq reads were aligned to house fly genome assembly v2.0.2 and annotation release 100 (Scott et al. 2014) using TopHat2 v2.0.8b (Kim et al. 2013) and Bowtie v2.1.0.0 (Langmead et al. 2009) with the default parameters. We tested for differential expression between males and females and between Y^M and III^M males using the Cuffdiff program in the Cufflinks v2.2.1 package (Trapnell et al. 2013) with the default parameters, including geometric normalization. We used a false discovery rate (FDR) corrected *P* value of 0.05 to identify genes that are differentially expressed (Benjamini and Hochberg 1995). Genes were considered not differentially expressed if Cuffdiff returned an “OK” value for the test status (at least ten reads aligned to the transcript, and data were sufficient for testing for differential expression) but the expression levels were not significantly different. Genes without an “OK” value were not included in downstream analyses. We repeated this analysis by also requiring a 2-fold difference in expression to call genes as differentially expressed. In comparisons between male and female expression levels, we treated

all six male samples as biological replicates and did the same for both female samples. We also repeated the analysis using only two replicates of each sample to control for sample-size effects.

We used expression level estimates from Cuffdiff2 (Fragments Per Kilobase of transcript per Million mapped reads) to calculate correlations of expression levels between our experimental samples (supplementary fig. S1, Supplementary Material online). Only genes with an “OK” value for test status in Cuffdiff were included. The correlations between testis and ovary expressions are lowest, which is expected because they are dramatically different tissues. The correlations between male and female head samples are substantially higher than between testis and ovary, but still lower than the correlations within sexes. The two ovary samples are more highly correlated than any of the pairwise comparisons between CS and CSaY testis samples (supplementary fig. S1, Supplementary Material online), most likely because CS and CSaY females are genetically identical (fig. 1). All data analysis was performed in the R statistical programming environment (R Core Team 2015).

Chromosomal Assignments of House Fly Genes

The house fly genomic scaffolds have not formally been assigned to chromosomes, but homologies have been inferred between house fly chromosomes and the five major chromosome arms of *Drosophila*, also known as Muller elements A–E (Foster et al. 1981; Weller and Foster 1993). Additionally, the house fly X chromosome is most likely homologous to the *Drosophila* dot chromosome (Muller element F, or *Drosophila melanogaster* chromosome 4) (Vicoso and Bachtrog 2013, 2015). We therefore assigned house fly genes that are conserved as one-to-one orthologs with *D. melanogaster* (Scott et al. 2014) to house fly chromosomes based on the Muller element mapping of the *D. melanogaster* orthologs. For our analysis of gene families that are differentially expressed between Y^M and III^M males, we assigned house fly scaffolds to chromosomes based on the Muller element mapping of the majority of *D. melanogaster* orthologs on each scaffold.

Gene Ontology Analysis

We used the predicted *D. melanogaster* orthologs (Scott et al. 2014) to infer the functions of house fly genes. Gene ontology (GO) annotations of house fly genes were determined using BLAST2GO (Conesa et al. 2005; Götz et al. 2008) as described previously (Scott et al. 2014). We then used BLAST2GO to identify GO classes that are enriched among differentially expressed genes relative to nondifferentially expressed genes using an FDR-corrected Fisher’s exact test (FET) (Benjamini and Hochberg 1995).

Quantitative Polymerase Chain Reaction Validation of Differentially Expressed Genes

We used quantitative polymerase chain reaction (qPCR) on cDNA to validate the differential expression of four genes between Y^M and III^M testes. Dissections of testes were performed as described above on three batches of Y^M and III^M males each (i.e., three biological replicates of each strain). RNA was extracted from testes using TRIzol homogenization followed by purification using Direct-zol RNA MiniPrep columns (Zymo Research). We synthesized cDNA using M-MLV reverse transcriptase (Promega) with oligo-dT primers following the manufacturer’s instructions. We designed PCR primers to amplify a 71–110 bp product at the 3’-end of each transcript, with one primer on either side of the 3’-most annotated intron when possible (supplementary table S1, Supplementary Material online). We also designed primers to amplify one transcript that was not differentially expressed between Y^M and III^M males or between males and females in either gonad or head in our mRNA-Seq analysis (XM_005187313). We tested our primer pairs with PCR using testis cDNA as a template to validate that they amplify a single product.

We then performed qPCR on three technical replicates of five serial dilutions of 1/5 each using a 60°C annealing temperature on an Applied Biosystems StepOnePlus Real-Time PCR System with Power SYBR Green PCR Master Mix (Life Technologies) following the manufacturer’s instructions. We assigned a threshold cycle (C_T) to the qPCR curves, and we validated that the primer pairs gave a linear relationship between C_T and $-\log_{10}$ concentration. We next used the same reagents and equipment to perform qPCR using each primer pair on three technical replicates of the three biological replicates from both strains (18 samples total), with the samples interspersed on a 96-well plate to avoid batch effects. In addition to the 18 samples, each qPCR plate contained three technical replicates of a five-step 1/5 serial dilution (15 samples). Those 33 samples were amplified by qPCR using primers for a gene that was detected as differentially expressed using mRNA-Seq and the 33 samples were also amplified with primers in our control gene (XM_005187313), for a total of 66 reactions on a single plate. From each plate, we constructed a standard curve for each primer pair using the relationship between C_T and $-\log_{10}$ concentration from our serial dilutions, and we used the slope of these lines to estimate the initial concentration of our template cDNA in each sample. We divided the initial concentration for our experimental gene by the estimated concentration for the control gene to determine a relative concentration for the experimental gene for each of the technical replicates.

To test for differential expression between Y^M and III^M samples, we first constructed a linear model with replicates nested within strains predicting the relative concentration of the gene in the R statistical programming environment (R Core Team 2015). We then used Tukey’s Honest Significant Differences

method to perform an analysis of variance to determine whether there is a significant effect of strain on expression level.

Results

Genes on the House Fly Third Chromosome Are More Likely to Be Differentially Expressed between Y^M and III^M Males than Genes on Other Autosomes

We used mRNA-Seq to compare gene expression in heads and gonads of house fly males and females of a Y^M strain (CSaY) and a III^M strain (CS). Males of the III^M strain are XX and heterozygous for the III^M chromosome and a standard third chromosome without M (fig. 1A). Males of the Y^M strain are XY (with the same X as the III^M strain) and homozygous for the standard third chromosome found in the III^M strain (fig. 1B). The rest of the genome is isogenic, and females of the two strains are genetically identical (fig. 1).

We detected 873 and 1,338 genes that are differentially expressed between Y^M and III^M males in heads or testes, respectively, at an FDR-corrected P value of 0.05 (table 1 and supplementary fig. S2 and supplementary data, Supplementary Material online). Genes on the house fly third chromosome are more likely than genes on other autosomes to be differentially expressed between Y^M and III^M males (fig. 2A). Approximately 30% of the differentially expressed genes are predicted to be on the third chromosome, which is greater than the fraction assigned to any of the other four autosomes (14.8–20.6%). There is a slight, but significant, signal of higher expression from the third chromosome in Y^M males when compared with III^M males (fig. 2B), and a significant excess of third chromosome genes is upregulated in Y^M males (supplementary fig. S3, Supplementary Material online). However, a significant excess of third chromosome genes is also upregulated in III^M males (supplementary fig. S3, Supplementary Material online), suggesting that the differential expression of third chromosome genes between Y^M and III^M males is not the result of degeneration of the III^M chromosome.

X-linked genes also trend toward an excess that are differentially expressed between Y^M and III^M males (fig. 2A), but we do not have the power to detect statistically significant deviations from the expectation because only 56 X-linked genes are expressed in head and 52 X-linked genes are expressed in testis. Surprisingly, expression from the X chromosome is higher in Y^M (XY) than III^M (XX) male heads (fig. 2B), and a significant excess of X-linked genes is upregulated in Y^M heads relative to III^M heads (supplementary fig. S3, Supplementary Material online). These results demonstrate that differential expression of X-linked genes between Y^M and III^M males is not the result of a dosage deficiency in hemizygous males. In addition, these patterns suggest that either a dosage compensation mechanism provides greater than 2-fold

upregulation of the X chromosome in XY males or *trans* effects of the Y chromosome lead to upregulation of X-linked expression.

Our chromosomal assignments of house fly genes are almost certainly less than perfect because of gene movement between chromosomes since the divergence between *D. melanogaster* the *M. domestica* lineages. However, errors in chromosomal assignments should obstruct the signal of elevated expression divergence on the third and X chromosomes, making our results conservative.

More Differential Expression between Y^M and III^M Males in Testis than in Head, but a Common Set of Genes Coregulated in Both Tissues

A higher fraction of genes is differentially expressed in testes between Y^M and III^M males than in heads (table 1; $P < 10^{-16}$ in FET), suggesting that genes involved in male fertility phenotypes are more affected by the M -bearing chromosome. When we restricted the analysis to only genes expressed in both heads and gonads, we still observe more genes differentially expressed in testes than heads between Y^M and III^M males (supplementary table S2, Supplementary Material online). When we used a 2-fold cutoff in addition to an FDR-corrected $P < 0.05$ cutoff, the number of genes differentially expressed in head and testis between Y^M and III^M males goes down to 373 and 558, respectively. However, there is still a higher fraction of genes differentially expressed in testis than head ($P_{\text{FET}} < 10^{-5}$).

Genes that are differentially expressed between males and females are said to have “sex-biased” expression (Ellegren and Parsch 2007). The fraction of genes differentially expressed between the testes of Y^M and III^M males is nearly as large as the fraction with sex-biased expression in head (table 1). We have a different number of replicates for male samples (three Y^M and three III^M , for a total of six male replicates) than female samples (two), and so our power to detect differential expression may differ between the interstrain (Y^M vs. III^M) and intersex comparisons. To control for sample size effects, we repeated our analysis using only two replicates of each male strain in the interstrain comparison and two male replicates (one from each strain) in the intersex comparison. With only two replicates of each sample we confirmed that more genes are differentially expressed between Y^M and III^M testes than heads, and we found that more genes are differentially expressed between Y^M and III^M males in either tissue than have sex-biased expression in head (supplementary table S3, Supplementary Material online). This result confirms that the interstrain expression differences are of a similar or greater magnitude than the amount of sex-biased expression in head.

If the probability that a gene is differentially expressed between Y^M and III^M male heads is independent of the probability that the gene is differentially expressed in testes, we expect less than 1% of genes to be differentially expressed

in both head and testis. We find that 176 genes (2.12%) are differentially expressed between Y^M and III^M males in both head and testis when using an FDR-corrected P value to test for differential expression, which is significantly greater than

Table 1
Differential Expression between Strains and Sexes

Tissue	Comparison	# Diff	# Nondiff	# Genes	Freq Diff
Male head	Y^M versus III^M	Y^M	III^M	9,441	0.092
		320	553		
Testis	Y^M versus III^M	Y^M	III^M	10,275	0.130
		782	556		
Head	Male versus female	male	female	9,679	0.133
		205	1,087		
Gonad	Testis versus ovary	male	female	10,586	0.736
		3,426	4,369		

NOTE.—Counts of the number of genes that are differentially expressed (# Diff), tested and nondifferentially expressed (# Nondiff), and total genes tested (# Genes), as well as the frequency of genes that are differentially expressed (Freq Diff) are listed in the table. For the # Diff columns, the number of genes up-regulated in each of the two samples being compared is presented.

the expectation ($P_{FET} < 10^{-25}$). In contrast, there is not a significant excess of genes with sex-biased expression in both head and gonad—we expect 9.41% of genes to have sex-biased expression in both head and gonad (supplementary table S2, Supplementary Material online), and we observe that 809 genes (9.27%) are sex-biased in both tissue samples ($P_{FET} = 0.655$). We obtain qualitatively similar results when using a 2-fold cutoff in addition to an FDR-corrected P -value threshold of 0.05 to test for differential expression: There is a 4-fold excess of genes that are differentially expressed in both head and testis between Y^M and III^M males ($P_{FET} < 10^{-26}$), and a less than 10% excess of genes that are differentially expressed in both head and gonad between males and females ($P_{FET} = 0.036$). We also get the same result when analyzing only two replicates of each sample: A significant excess of genes is differentially expressed in both head and testis between Y^M and III^M males ($P_{FET} < 10^{-4}$), but not between males and females in both head and gonad ($P_{FET} > 0.3$). These results suggest that there are many genes under common regulatory control by the M -bearing chromosome in both male head and testis, but there is not the same degree of sex-specific regulation in common between head and gonad.

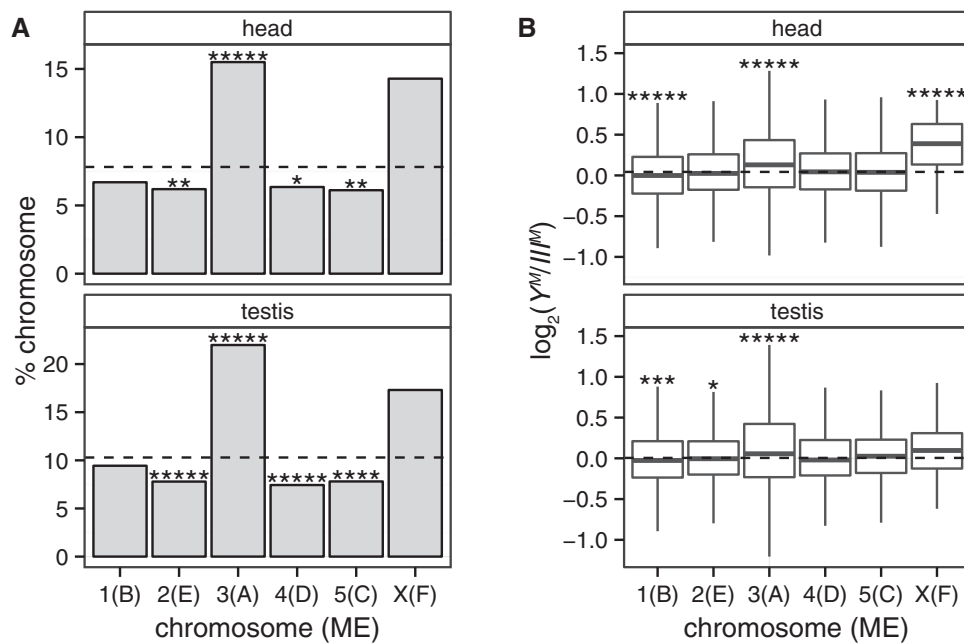


Fig. 2.—Chromosomal mapping and differential expression between Y^M and III^M males. (A) Bar graphs indicate the percent of genes on each chromosome (*Drosophila* Muller element in parentheses) that are differentially expressed between Y^M and III^M male heads (top) or testes (bottom). The dashed line indicates the percentage of genes that are differentially expressed across all chromosomes. Asterisks indicate P values from FET comparing the number of differentially expressed genes with the number not differentially expressed on a chromosome and summed across all other chromosomes ($*P < 0.05$, $**P < 0.005$, $***P < 0.00005$, $****P < 0.000005$). (B) Box plots show the relative expression levels of genes in Y^M and III^M males on each chromosome. Expression level was measured in head (top) and testis (bottom). The dashed line indicates the average \log_2 expression ratio across all genes. Asterisks indicate P values from a Mann–Whitney test comparing the expression ratio between genes on one chromosome versus the rest of the genome ($*P < 0.05$, $**P < 0.005$, $***P < 0.0005$, $****P < 0.00005$, $*****P < 0.000005$).

Genes That Are Differentially Expressed between Y^M and III^M Males Are More Likely to Have Male-Biased Expression

Genes whose expression is significantly higher in males than females are said to have “male-biased” expression, and genes that are upregulated in females have “female-biased” expression (Ellegren and Parsch 2007). We found that genes with male-biased expression in head are more likely to be differentially expressed between Y^M and III^M male heads than genes with either female-biased or unbiased expression (fig. 3A). Similarly, genes that are upregulated in testis relative to ovary (testis-biased) are more likely to be differentially expressed between Y^M and III^M testes than genes with “ovary-biased” or unbiased expression in gonad (fig. 3B). We repeated this analysis using two replicates of each sample, and we consistently observe that genes with male-biased expression in head or gonad are more likely to be differentially expressed between Y^M and III^M males (supplementary figs. S4 and S5, Supplementary Material online).

We also found that 14.8% of genes that are upregulated in III^M male heads have male-biased expression, whereas less than 2% of genes that are upregulated in Y^M male heads have male-biased expression ($P_{FET} < 10^{-10}$). We observe the same excess of male-biased genes upregulated in III^M male heads when we only use two replicates of each strain and sex to test for differential expression ($P_{FET} < 0.05$ using most combinations of two replicates). This suggests that III^M male heads have a “masculinized” expression profile relative to Y^M heads.

Functional Annotations of Genes That Are Differentially Expressed between Y^M and III^M Males

We tested for GO categories that are overrepresented among genes with sex-biased expression that are differentially expressed between Y^M and III^M males (supplementary data, Supplementary Material online). We found that nearly half (49.7%) of genes that are differentially expressed between Y^M and III^M male heads are annotated with the functional category “catalytic activity,” whereas only 43% of genes not differentially expressed have that GO annotation ($P_{FET} < 0.05$ corrected for multiple tests). Over 10% of the genes with the catalytic activity annotation that are upregulated in III^M male head are predicted to encode proteins involved in a metabolic process, including metabolism of organic acids, amino acids, and lipids. Among those genes, 15 are annotated as cytochrome P450 (CYP450) genes, and 4 of those also have male-biased expression in head (supplementary table S6, Supplementary Material online). CYP450s collectively carry out a wide range of chemical reactions including metabolism of endogenous (e.g., steroid hormones) and exogenous (e.g., xenobiotics) compounds (Scott 2008). All 15 differentially expressed CYP450s are upregulated in III^M males, and no CYP450 genes are upregulated in Y^M males. Five of the CYP450s are on scaffolds that we assign to the third chromosome (supplementary table S6, Supplementary Material online), suggesting that *cis* regulatory sequences controlling the expression of CYP450s have diverged between III^M and the standard third chromosome. However, five of the

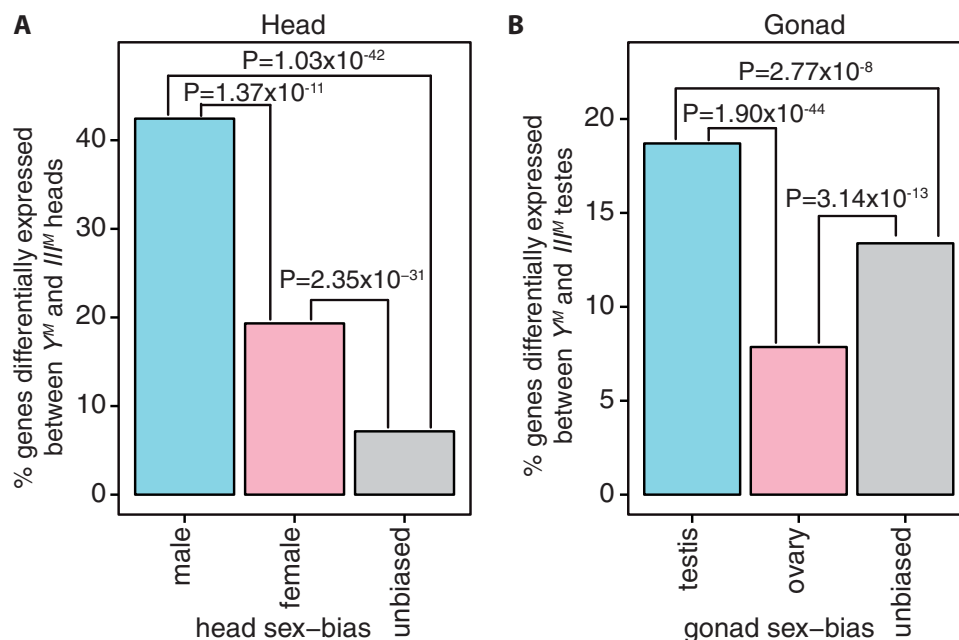


FIG. 3.—Sex-biased expression of genes differentially expressed between Y^M and III^M males. Bar graphs indicate the percentage of genes with male-biased (blue), female-biased (pink), or unbiased (gray) expression in either (A) head or (B) gonad that are differentially expressed between Y^M and III^M males in either (A) head or (B) testis. *P* values are for FET between groups.

CYP450s can be assigned to other autosomes (the remaining five cannot be assigned to a chromosome), demonstrating that divergence of *trans*-factors between *III^M* and the standard third chromosome is also responsible for differential expression of CYP450s between *Y^M* and *III^M* males. The 15 CYP450s represent a range of different clans (2–4) and families (4, 28, 304, 313, 438, and 3073) (Scott et al. 2014). However, an excess of CYP450s from clan 4 are upregulated in *III^M* male head ($\chi^2 = 4.19$, $P = 0.041$), and thus overexpression of CYP450s is not random.

Genes that are annotated as encoding proteins located in extracellular regions are overrepresented among genes with testis-biased expression (15.0% of genes with testis-biased expression; 9.9% of genes not differentially expressed between testis and ovary; $P_{\text{FET}} < 10^{-3}$ corrected for multiple tests) and among genes that are differentially expressed between *Y^M* and *III^M* testes (13.9% of differentially expressed genes; 8.1% of non-differentially expressed genes; $P_{\text{FET}} < 10^{-4}$ corrected for multiple tests). In addition, 3.1% of the genes differentially expressed between *Y^M* and *III^M* testes are predicted to encode carbohydrate-binding proteins (compared with 1.4% of nondifferentially expressed genes; $P_{\text{FET}} < 0.05$ corrected for multiple tests), and 7.2% of differentially expressed genes are predicted to encode structural molecules (compared with 3.7% of nondifferentially expressed genes; $P_{\text{FET}} < 10^{-3}$ corrected for multiple tests). Three of those structural molecules are predicted to be β -tubulin proteins encoded by genes that are upregulated in *Y^M* testes relative to *III^M*, and two of those genes also have testis-biased expression. We tested for differential expression of the two of the β -tubulin genes with testis-biased expression using qPCR (supplementary fig. S6 and supplementary data, Supplementary Material online). Only one of the two (XM_005187368) was upregulated in *Y^M* testis when assayed with qPCR ($P < 10^{-4}$), whereas the other gene (XM_005175742) was not ($P = 0.653$) possibly because of high variance in the *Y^M* measurement (supplementary fig. S6, Supplementary Material online). The *D. melanogaster* genome encodes a testis-specific β -tubulin paralog that is essential for spermatogenesis (Kemphues et al. 1982; Hoyle and Raff 1990), suggesting that the β -tubulin gene that is upregulated in *Y^M* testis may be important for sperm development.

Four genes that are differentially expressed between *Y^M* and *III^M* testes are homologs of the *D. melanogaster* Y-linked fertility factors *kl-2*, *kl-3*, and *kl-5* (Goldstein et al. 1982; Gepner and Hays 1993; Carvalho et al. 2000, 2001). These proteins encode components of the dynein heavy chain, which is necessary for flagellar activity of sperm. All four of the predicted dynein heavy chain genes that are differentially expressed between *Y^M* and *III^M* testes are autosomal in house fly. Three of these genes have testis-biased expression—two of those are upregulated in *III^M* testis (XM_005175130 and XM_005176585), whereas the third is upregulated in *Y^M* testis (XM_005184828). The fourth gene

(XM_005184771) is upregulated in *III^M* testis, but it is not differentially expressed between testis and ovary. Using qPCR, we validated that XM_005184828 is upregulated in *Y^M* testis ($P < 10^{-4}$) and XM_005176585 is upregulated in *III^M* testis ($P < 0.01$) (supplementary fig. S6 and supplementary data, Supplementary Material online). Two additional genes encoding components of other dynein chains have testis-biased expression and are upregulated in *III^M* testis relative to *Y^M* testis.

Finally, there are numerous predicted RNAs in the house fly genome annotation that have no identifiable homology to any known RNAs or proteins (Scott et al. 2014). We identified six of these uncharacterized RNAs that both have testis-biased expression and are differentially expressed between *Y^M* and *III^M* testes. XR_225504, XR_225520, and XR_225639 are upregulated in *Y^M* testes, and XR_225442, XR_225497, and XR_225737 are upregulated in *III^M* testes. These genes are annotated as encoding noncoding RNAs (ncRNAs), and we were unable to detect long open-reading frames in the transcripts. It is possible that these ncRNAs are responsible for the regulation of gene expression in testis, and differential expression of these ncRNAs between *Y^M* and *III^M* testis could be responsible for the differential expression of other genes between *Y^M* and *III^M* males.

Discussion

Differential Expression between *Y^M* and *III^M* Males Is Driven by Both *Cis* and *Trans* Effects

We compared gene expression in head and testis between *Y^M* and *III^M* males. *Y^M* males are homozygous for a standard third chromosome that does not have *M*, whereas *III^M* males are heterozygous for a *III^M* chromosome and a standard third chromosome (fig. 1). Differences in the expression levels of autosomal genes between *Y^M* and *III^M* males could be the result of 1) divergence of *cis*-regulatory sequences between the *III^M* and standard third chromosomes that affect the expression of genes on the third chromosome, 2) divergence of *trans*-factors between *III^M* and the standard third chromosome that differentially regulate gene expression throughout the genome, 3) downstream effects of the first two processes that lead to further differential expression.

The two strains also differ in the genotype of their sex chromosomes; *Y^M* males are XY, whereas *III^M* males are XX (fig. 1). The house fly Y chromosome is highly heterochromatic and does not harbor any known genes other than *M* (Boyes et al. 1964; Hediger et al. 1998; Dübendorfer et al. 2002). It is clear that the Y chromosome does not contain any genes necessary for male fertility or viability because XX males are fertile and viable. The X chromosome is also highly heterochromatic and probably homologous to the *Drosophila* dot chromosome (Hediger et al. 1998; Vicoso and Bachtrög 2013, 2015). The heterochromatic *Drosophila* Y chromosome can affect the expression of autosomal genes (Lemos et al.

2008, 2010; Sackton et al. 2011; Zhou et al. 2012), suggesting that the house fly *X* and *Y* chromosomes could have *trans* regulatory effects on autosomal gene expression.

A higher fraction of third chromosome genes are differentially expressed between Y^M and III^M house fly males than genes on any other autosome (fig. 2A). Therefore, divergence of *cis*-regulatory sequences between III^M and the standard third chromosome is at least partially responsible for the expression differences between Y^M and III^M males. However, approximately 70% of the genes differentially expressed between Y^M and III^M males map to one of the other four autosomes, suggesting that the majority of expression differences is the result of *trans* effects of the *X*, *Y*, and third chromosomes along with further downstream effects.

Reproductive and Male Phenotypes Are More Likely to Be Affected by *M* Variation

Reproductive traits are more sexually dimorphic than nonreproductive traits, and reproductive traits also tend to evolve faster, possibly as a result of sexual selection (Eberhard 1985). A similar faster evolution of gene expression in reproductive tissues has been observed across many taxa (Khaitovich et al. 2005; Zhang et al. 2007; Brawand et al. 2011), and increased variation within species for sex-biased gene expression often accompanies elevated expression divergence (Meiklejohn et al. 2003; Ayroles et al. 2009). Consistent with these patterns, more genes are differentially expressed between Y^M and III^M males in testis than head (table 1 and supplementary fig. S2, Supplementary Material online). Somatic and germline SDs in house fly are under the same genetic control (Hilfiker-Kleiner et al. 1994), so the exaggerated differences in expression between Y^M and III^M testes relative to heads cannot be attributed to differences in the SD pathway between gonad and head. We also find that genes with male-biased expression are more likely to be differentially expressed between Y^M and III^M males (fig. 3). Genes with male-biased expression are more likely to perform sex-specific functions (Connallon and Clark 2011), suggesting that genes that are differentially expressed between Y^M and III^M males disproportionately affect male phenotypes.

Evaluating the Role of Sex-Specific Selection in MSD Turnover

Many models of SD evolution predict that a new MSD locus will invade a population if it is genetically linked to an allele with a beneficial, sexually selected, or sexually antagonistic fitness effect (Charlesworth D and Charlesworth B 1980; Rice 1987; Charlesworth 1991, 1996; Rice, 1996; van Doorn and Kirkpatrick 2007, 2010). Alternatively, evolutionary turnover of MSD loci could be the result of neutral drift in a highly labile system (van Doorn 2014).

Our results are consistent with a model in which the III^M chromosome invaded because it harbors alleles with

male-specific beneficial effects. First, the expression of genes that are likely to perform male-specific functions—especially in male fertility—is more likely to be affected by the III^M chromosome (table 1; fig. 3), and those male-specific phenotypic differences could have been targets of sex-specific selection pressures. However, as mentioned above, the expression of male-biased genes is more variable than other genes even in species without multifactorial SD systems (Meiklejohn et al. 2003; Ayroles et al. 2009). Additional experiments in which a non-*M*-bearing chromosome is placed on a common genetic background are therefore necessary to further test the hypothesis that the *M*-bearing chromosome disproportionately affects male-biased gene expression.

We also found that III^M heads have a masculinized expression profile relative to Y^M heads, suggesting that the male-limited transmission of the III^M chromosome favored the accumulation of alleles with male-beneficial fitness effects (Rice 1984). Previous work found that III^M males outperformed Y^M males in multiple laboratory fitness assays (Hamm et al. 2009), providing additional support for the accumulation of male-beneficial alleles on the III^M chromosome. However, despite the apparent selective advantage of the III^M chromosome, it surprisingly does not appear to be expanding rapidly (Hamm et al. 2015), suggesting that the fitness benefits of III^M may be environment-specific (Feldmeyer et al. 2008).

Our data do not allow us to distinguish between two possible orders of events in the invasion of the III^M chromosome. In the first scenario, male-beneficial alleles on the third chromosome could have driven the initial invasion of III^M (van Doorn and Kirkpatrick 2007). In the second scenario, beneficial alleles could have accumulated on the III^M chromosome after it acquired an *M*-locus because male-limited inheritance promotes the fixation of male-beneficial alleles (Rice 1984, 1987). These scenarios are not mutually exclusive. Regardless of the sequence of events, we have provided evidence that the house fly multifactorial male-determining system is associated with phenotypic differences that likely have male-specific fitness effects, which could explain the invasion of the III^M chromosome under sexual or sexually antagonistic selection.

Supplementary Material

Supplementary data, figures S1–S6, and tables S1–S6 are available at *Genome Biology and Evolution* online (<http://www.gbe.oxfordjournals.org/>).

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