Minimal effects of proto-Y chromosomes on house fly gene expression in spite of evidence that selection maintains stable polygenic sex determination

Jae Hak Son[1], Tea Kohlbrenner[2], Svenia Heinze[2], Leo Beukeboom[3], Daniel Bopp[2], Richard P. Meisel[1]

1. Department of Biology and Biochemistry, University of Houston, USA

2. Institute of Molecular Life Sciences, University of Zurich, Switzerland

3. Groningen Institute for Evolutionary Life Sciences, University of Groningen, The Netherlands

Abstract

Sex determination, the developmental process by which organismal sex is established, evolves fast, often due to changes in the master regulators at the top of the pathway. Additionally, in species with polygenic sex determination, multiple different master regulators segregate as polymorphisms. Understanding the forces that maintain polygenic sex determination can be informative of the factors that drive the evolution of sex determination. The house fly, *Musca domestica*, is a well-suited model to those ends because natural populations harbor male-determining loci on each of the six chromosomes and a bi-allelic female-determiner. To investigate how natural selection maintains polygenic sex determination in house fly, we assayed the phenotypic effects of proto-Y chromosomes by performing RNA-seq experiments to measure gene expression in house fly males carrying different proto-Y chromosomes. We find that the proto-Y chromosomes have similar effects as a non-sex-determining autosome. In addition, we created sex-reversed males without any proto-Y chromosomes, and they had nearly identical gene expression profiles as genotypic males. Therefore, the proto-Y chromosomes have a minor effect on male gene expression, consistent with previously described minimal X-Y sequence differences. Despite these minimal differences, we find evidence for a disproportionate effect of one proto-Y chromosome on male-biased expression, which could be partially responsible for fitness differences between males with different proto-Y chromosome genotypes. Our results therefore suggest that, if natural selection maintains polygenic sex determination in house fly via gene expression differences, the phenotypes under selection likely depend on a small number of genetic targets.

Introduction

Sex determination is the process by which genetic or environmental cues cause an individual to develop into either a female or male. Sex determination evolves rapidly, often due to changes in the master regulatory genes at the top of sex determining pathways (Beukeboom and Perrin 2014). Sex determining pathways can also be variable (polygenic or multifactorial) within species (Moore and Roberts 2013). Many population genetic models predict that polygenic sex determination should be a transient state between monogenic equilibria (Rice 1986; van Doorn and Kirkpatrick 2007). It is therefore surprising that polygenic sex determination has been found in numerous species (Orzack *et al.* 1980; Moore and Roberts 2013; Bachtrog *et al.* 2014). Understanding how polygenic sex determining systems are maintained will help shed light on the forces driving the rapid evolution in sex determination pathways.

The house fly, *Musca domestica*, is a model species to study polygenic sex determination because it has a well characterized and highly variable sex determination system (Dübendorfer *et al.* 2002; Hamm *et al.* 2015). The male-determining gene, *Mdmd*, appears to be recently derived in house fly as it is absent in its close relative *Stomoxys calcitrans*, and it cannot be found in other related dipterans (Sharma *et al.* 2017). *Mdmd* regulates the splicing of the house fly ortholog of *transformer* (*Md-tra*), preventing males from producing a functional female-determining isoform of *Md-tra* (Hediger *et al.* 2010; Sharma *et al.* 2017). A dominant female-determining allele (*Md-tra*^{D}) that is not sensitive to *Mdmd* regulation also segregates in natural populations, allowing females to carry *Mdmd* (McDonald *et al.* 1978; Kozielska *et al.* 2008; Hediger *et al.* 2010).

Mdmd can be found on multiple different chromosomes in natural populations of house fly (Sharma *et al.* 2017). The two most common locations of *Mdmd* in natural populations are the Y chromosome (Y^M) and third chromosome (III^M) (Hamm *et al.* 2015). Historically, the chromosomes carrying the male determiner were designated as the Y (Y^M), X (X^M), and any of the five autosomes (e.g., III^M). However, recent work showed that the Y^M chromosome is highly similar in gene content to the X chromosome because Y^M is a very young proto-Y chromosome (Meisel *et al.* 2017). These findings align with the independent discovery that *Mdmd* is of a recent origin (Sharma *et al.* 2017). Moreover, previous work observed minimal morphological and sequence differences between the X and Y chromosomes (Boyes *et al.* 1964; Hediger *et al.* 1998). The third chromosome carrying *Mdmd* is also very recently derived from the standard third chromosome (Meisel *et al.* 2017). We therefore refer to any chromosome carrying *Mdmd* (including Y^M and III^M) as a proto-Y chromosome.

There are multiple lines of evidence that natural selection maintains polygenic sex determination in house fly. First, Y^M and III^M form stable latitudinal clines on multiple continents (Franco *et al.* 1982; Tomita and Wada 1989; Hamm *et al.* 2005; Kozielska *et al.* 2008). Y^M is most frequent in northern populations, and III^M predominates in southern populations. The distributions of the proto-Y chromosomes correlate with seasonality in temperature (Feldmeyer *et al.* 2008), suggesting that temperature modulates the fitness of males carrying different proto-Y chromosomes. Second, males carrying Y^M or III^M differ in their success courting female mates, and the frequency of the III^M chromosome reproducibly increased over generations in laboratory population cages kept at a warm temperature (Hamm *et al.* 2009). Third, in some populations,

individual males carry multiple proto-Y chromosomes, which would cause them to produce male-biased broods with their mates (Kozielska *et al.* 2006; Hamm *et al.* 2015). The frequency of males that carry multiple proto-Y chromosomes is positively correlated with the frequency of *Md-tra*^{D} across populations (Meisel *et al.* 2016). This suggests that *Md-tra*^{D} invaded to balance the sex ratio or *Md-tra*^{D} allows for the increase in frequency of proto-Y chromosomes.

If natural selection maintains polygenic sex determination in house fly, Y^{M} and III^{M} must have different phenotypic and fitness effects for selection to act upon. However, a recent analysis of Y^{M} and III^{M} sequences revealed very few differences from their homologous X and III chromosomes, respectively (Meisel *et al.* 2017). To examine this paradox of evolutionarily important phenotypic effects of proto-Y chromosomes yet minimal sequence divergence from their homologs, we used high throughput mRNA sequencing (RNA-seq) to measure gene expression in house flies with different Y^{M} and III^{M} genotypes. This included testing the effects of multiple different naturally occurring Y^{M} and III^{M} chromosomes on a common genetic background. We also used RNA interference (RNAi) to knock down *Md-tra* and create sex-reversed males that do not carry any proto-Y chromosomes (Hediger *et al.* 2010), which we compared to males with the same genetic background carrying III^{M}. Our experiments therefore allow us to identify candidate phenotypic differences between males carrying different proto-Y chromosomes upon which natural selection can act to maintain polygenic sex determination in house fly.

Materials and Methods

Strains with naturally occurring proto-Y chromosomes

We examined gene expression in four house fly strains that each have a different naturally occurring proto-Y chromosome (either Y^{M} or III^{M}) on a common genetic background (Figure 1). We used a previously described backcrossing method to move each proto-Y chromosome onto the Cornell Susceptible (CS) genetic background (Meisel *et al.* 2015). CS is an inbred III^{M} strain produced by mixing strains collected from throughout the United States (Scott *et al.* 1996). Our first proto-Y chromosome is the III^{M} chromosome from the CS strain on its native background. The second strain (CSrab) was created by backcrossing the III^{M} chromosome from the rspin strain collected in New York (Shono and Scott 2003) onto the CS background for 10 generations, replacing the CS III^{M} chromosome (Supplementary Figure 1). The third strain (IsoCS) is a Y^{M} strain that was previously created by introducing the Y^{M} chromosome from a strain collected in Maine onto the CS background without III^{M} (Hamm *et al.* 2009). The fourth strain was created to test the effect of a non-*Mdmd*-bearing third chromosome on gene expression (Supplementary Figure 2). To that end, we introduced the third chromosome carrying the recessive *brown body* mutation (*bwb*) and the Y^{M} chromosome from the genome reference strain (aabys) onto the CS background to create the bwbCS strain (III^{bwb}/ III^{bwb}; X/Y^{M}). We then crossed bwbCS males with CS females (bwbCS×CS) to create males that carry the aabys Y^{M} chromosome and are heterozygous for the non-*Mdmd* third chromosomes from CS and aabys on a CS background (III^{CS}/ III^{bwb}; X^{CS}/Y^{M}). We therefore have two III^{M} strains (CS and CSrab) with different origins of the III^{M} chromosome and two Y^{M} strains (IsoCS and bwbCS×CS) with different origins of the Y chromosome. In three of the strains (CS, CSrab, and IsoCS), females are isogenic for the CS

128 background and males are isogenic except for their *Mdmd*-bearing proto-Y chromosomes.
129
130 **RNAi knockdown to create sex reversed flies**
131
132 We used RNAi targeting *Md-tra* to create sex-reversed males that do not carry a male-
133 determining proto-Y chromosome. For the RNAi experiments we used a house fly strain that
134 allows easy identification of sex-reversed individuals that are genotypic females but phenotypic
135 males (Hediger *et al.* 2010). Females of this strain are homozygous for a third chromosome
136 containing recessive alleles for *pointed wing* (*pw*) and *bwb*. Males carry one copy of the third
137 chromosome with *pw* and *bwb*, and one copy of a III^M chromosome with wild-type alleles
138 (*Mdmd* pw^+ bwb^+/*pw bwb*). Females therefore have pointed wings and brown bodies, as do sex-
139 reversed males, whereas normal males have wild-type wings and wild-type bodies. We cannot do
140 this same experiment with Y^M males because there are no morphological markers on the X/Y
141 chromosome.
142
143 Double-stranded RNA (dsRNA) targeting *Md-tra* (*Md-tra*-RNAi) and GFP (GFP-RNAi) was
144 generated and injected into early blastoderm embryos of the *pw bwb* strain following established
145 protocols (Hediger *et al.* 2001, 2004). The fragment of dsRNA targeting *Md-tra* ranges from
146 exon 1 to exon 5, and it was generated by amplifying cDNA from female house flies (Hediger *et*
147 *al.* 2010). The sequences of the T7 extended primers used to produce dsRNA targeting *Md-tra*
148 are 5'-gtaatacgatcactatagggTGGTGTAATATGGCTCTATCG-3' and 5'-
149 gtaatacgatcactatagggGCTGCCATACAAACGTGTC-3' (sequences in lower case are the T7
150 region and sequences in upper case anneal to *Md-tra*). The sequences of the T7 extended primers
151 used to produce dsRNA targeting GFP are 5'-gtaatacgatcactatagggATGTGAGCAAGGGC-3'
152 and 5'-gtaatacgatcactatagggCTTGTACAGCTCGTC-3'.
153
154 The larvae that hatched from embryos injected with either *Md-tra*-RNAi or GFP-RNAi were
155 raised on porcine feces because the small number of injected larvae are less likely to develop into
156 adult flies on standard rearing media (Schmidt *et al.* 1997). Under the injection scheme (Table
157 1), we could collect four types of flies: (A) genotypic females with the GFP-RNAi treatment
158 (phenotypic females), (B) genotypic females with the *Md-tra*-RNAi treatment (sex-reversed
159 males), (C) genotypic males with GFP-RNAi treatment (III^M males #1), and (D) genotypic males
160 with the *Md-tra*-RNAi treatment (III^M males #2). Both types of genotypic males (III^M males #1
161 and #2) are also phenotypic males, and the GFP-RNAi treated genotypic females are phenotypic
162 females. Sex reversal to a phenotypic male occurs in genotypic females under the *Md-tra*-RNAi
163 treatment (Hediger *et al.* 2010).
164
165 After emergence from pupa, each injected single phenotypic male was kept in a small cage with
166 three or four females from the *pw bwb* strain that did not have any injection treatments. Only
167 phenotypic males that successfully sired offspring with those females were retained for the
168 RNA-seq experiment. All three types of phenotypic males produced offspring, but the sex-
169 reversed males sired only female offspring (because they do not carry *Mdmd*). To measure gene
170 expression in females, we collected virgin GFP-RNAi treated genotypic females. Those females
171 were collected within 8 hours of emergence and kept separate from males to ensure they were
172 virgin. The females were aged for five days, and we selected three females to dissect for RNA-
173 seq experiments. We measured gene expression in virgin females to exclude mating effects on

174 female gene expression.
175
176 **RNA-seq experiments**
177
178 We used RNA-seq to measure gene expression in heads and abdomens from individual males of
179 the four strains carrying either the Y^M or III^M proto-Y chromosomes (Figure 1). The larvae were
180 raised at 25˚C on a standard diet of wheat bran, calf manna, yeast, reptile litter, and water, as
181 described previously (Hamm *et al.* 2009; Meisel *et al.* 2015). Unmated adult males and females
182 were sorted within 8 hours of emergence, kept separately at 22˚C, and provided water, sugar, and
183 powdered milk *ad libitum*. Heads and abdomens from adult flies at five days post emergence
184 were dissected and frozen at -80˚C. The heads and abdomens from individual males were
185 homogenized in TRIzol Reagent, and then RNA was extracted using the Zymo Direct-zol kit
186 following the manufacturer's protocol including DNA digestion steps. Three biological
187 replicates (i.e., three individual male heads and abdomens) were prepared from males of each of
188 the four strains. Because females of three strains (CS, CSrab, and IsoCS) are isogenic, we
189 sampled only one female from each of the strains. However, the RNA-seq library preparation for
190 CS female abdomen failed, so that the female abdomen had only two biological replicates.
191
192 We also performed RNA-seq on heads and abdomens from the four types of flies injected with
193 dsRNA (Table 1). Individual four to five-day old adult flies (described above) were frozen in
194 liquid nitrogen, and RNA was extracted from the individual flies with the NucleoSpin RNAII kit
195 (Macherey-Nagel, Düren, Germany) following the protocol of the manufacturer, which includes
196 DNA digestion steps. Three biological replicates (i.e., three individual flies) from each of the
197 four genotype-by-treatment combinations were collected.
198
199 RNA-seq libraries were prepared with the Illumina TruSeq Stranded mRNA Sample Preparation
200 Kit following the protocols of the manufacturer. The libraries were run in six lanes for 75 cycles
201 (i.e., 75 nucleotide reads) on an Illumina NextSeq500 machine at University of Houston Seq-N-
202 Edit Core. For the strains with different naturally occurring proto-Y chromosomes (Figure 1),
203 two of three lanes contained ten libraries comprised of one replicate from each strain, sex, and
204 body part (four strains of males plus one of the strains of females by two tissues): CS male head
205 and abdomen, CSrab male head and abdomen, IsoCS male head and abdomen, bwbCS male head
206 and abdomen, and female head and abdomen. The third lane contained nine of the samples
207 described above, but no CS female abdomen because that library preparation failed. For the
208 RNAi experiment we ran three lanes, and each lane contained eight library samples, one replicate
209 from each genotype-by-treatment combination and body part: *Md-tra*-RNAi genotypic female
210 head and abdomen (sex-reversed male), *Md-tra*-RNAi genotypic male head and abdomen, GFP-
211 RNAi genotypic female head and abdomen, and GFP-RNAi genotypic male head and abdomen.
212
213 **Data analysis**
214
215 Illumina RNA-seq reads were aligned to house fly genome assembly v2.0.2 and annotation
216 release 102 (Scott *et al.* 2014) using HISAT2 v2.0.1 (Kim *et al.* 2015). Read mapping
217 information for the strains with different naturally occurring proto-Y chromosomes and flies
218 from the RNAi experiment are shown in Supplementary Tables 1 and 2. Read coverage across
219 the sex determining genes *Md-tra*, *doublesex* (*Md-dsx*), and *fruitless* (*Md-fru*) was determined

220 with the 'mpileup' function in SAMtools (Li *et al.* 2009). Second, the aligned reads were
221 assigned to all annotated genes with htseq-count in HTSeq v0.9.1 (Anders *et al.* 2015), with the -
222 -stranded=reverse option because we generated stranded RNA-seq libraries.
223
224 The HTSeq output was used as input into DESeq2 v1.16.1 to identify differentially expressed
225 genes (Love *et al.* 2014). For the DESeq2 analysis of the four strains with different naturally
226 occurring Y^M and III^M chromosomes, we performed pair-wise comparisons between males of
227 each strain. We also performed pair-wise comparisons of males from each strain against females.
228 For the RNAi experiment, we created a model in DESeq2 in which gene expression is predicted
229 by genotypic sex, RNAi treatment (GFP-RNAi or *Md-tra*-RNAi), and the interaction between
230 genotypic sex and RNAi treatment. The model allows for pair-wise comparisons between
231 individuals with either the same genotypic sex or RNAi treatment. From the pair-wise
232 comparisons, $\log_2$ fold-changes ($\log_2$FC) were extracted for each gene with false discovery rate
233 corrected *P* values (Benjamini and Hochberg 1995). We also extracted $\log_2$FC for III^M males #2
234 over females using the equation: $\log_2$(III^M males #2 / females) = $\log_2$(III^M males #1 / females) +
235 $\log_2$(III^M males #2 / III^M males #1). We cannot calculate a *P* value for a test of whether $\log_2$(III^M
236 males #2 / females) is different from zero because it is not a pair-wise comparison performed by
237 the model we created in DESeq2. Only genes with adjusted *P* values reported by DESeq2 are
238 presented and used for downstream analyses. In other words, we considered a gene to be
239 expressed if there was enough data to compare gene expression levels, and we ignored genes
240 where a statistical test was not performed because expression was too low.
241
242 We performed a principal component (PC) analysis, used a grade of membership model
243 implemented in the R package 'CountClust' (Dey *et al.* 2017), and performed hierarchical
244 clustering to analyze the normalized expression count data from DESeq2. For the PC analysis
245 (PCA) and hierarchical clustering, we used a regularized log transformation of the normalized
246 count data calculated using the 'rlog' function in DESeq2 (Love *et al.* 2014). Because genes with
247 low counts show the highest relative differences among samples and create large variances, these
248 low count genes dominate the results of the PCA. The regularized log transformation stabilizes
249 the variance of the data, making it homoscedastic. The hierarchical clustering was performed
250 using the pheatmap() function in R (Kolde 2019). Gene Ontology (GO) terms were analyzed
251 with DAVID v.6.8 (Huang *et al.* 2009).
252
253 We assigned house fly genes to chromosomes using the conservation of Muller elements across
254 flies (Foster *et al.* 1981; Weller and Foster 1993), as done previously (Meisel *et al.* 2015; Meisel
255 and Scott 2018). Briefly, the house fly and *Drosophila* genomes are organized into six
256 chromosome arms (Muller elements A-F). Elements A-E correspond to the house fly
257 chromosomes that were historically considered the autosomes. Element F is the historical house
258 fly X chromosome (Vicoso and Bachtrog 2013). One-to-one orthologs between house fly and
259 *Drosophila melanogaster* genes were identified as part of the house fly genome annotation (Scott
260 *et al.* 2014). We assigned house fly scaffolds to Muller elements using a "majority rules"
261 approach—if the majority of genes on a scaffold were orthologous to *D. melanogaster* genes on
262 a single Muller element, then the house fly scaffold was assigned to that Muller element. In turn,
263 all genes on that scaffold are assigned to the same Muller element.
264
265

Data Availability

All RNA-seq data are available from the NCBI Sequence Read Archive within BioProject accessions PRJNA522991 (GEO accession GSE126685) and PRJNA522995 (GEO accession GSE126689).

Results

Different proto-Y chromosomes have minor effects on gene expression

We previously observed that hundreds of genes are differentially expressed between males carrying III^M and males carrying Y^M (Meisel *et al.* 2015). Those experiments controlled for genetic background by introgressing a Y^M chromosome onto the same background as the III^M chromosome. However, the previous work did not compare the effects of the proto-Y chromosome with the effect of introducing a new autosome on the common genetic background. It is therefore not clear if the expression differences between Y^M and III^M males were specific to introducing a new proto-Y chromosome onto a genetic background, or if changing any single chromosome can induce similar expression effects. To address this question, we used RNA-seq to measure gene expression in males from four nearly isogenic strains carrying either a Y^M or III^M chromosome (Figure 1). Two strains with "III^M males" have different III^M chromosomes on a common genetic background. A third strain with "Y^M males" has a Y^M chromosome, instead of III^M, on the same genetic background. The fourth strain carries a different Y^M chromosome and a single copy of a different standard third chromosome (without *Mdmd*) on the same genetic background as the other three strains. If the III^M chromosome has a disproportionate effect on gene expression, we expect to observe more genes differentially expressed between III^M and Y^M males than between Y^M males that differ from each other by a single standard third chromosome.

To compare gene expression profiles across the strains, we used both a PCA and a grade of membership model (Dey *et al.* 2017). We excluded one of the three batches of male samples (one replicate from each of the four strains) in both abdomen and head because males in that batch had outlier expression profiles. Specifically, the expression profiles of males from the outlier batch did not cluster with the replicates with the same genotype from the other two batches in our PCA (Supplementary Figure 3). Similarly, in a grade of membership model, males from the outlier batch had dramatically different group assignments than males with the same genotype from the other two batches (Supplementary Figure 3).

After excluding the outlier batch, we find that sex explains much of the variance in gene expression in both abdomen and head. In abdomen, the first PC (PC1) and second PC (PC2) explain 84% and 7% of variance in gene expression across samples, respectively (Figure 2A). In head, PC1 and PC2 explain 43% and 28% of variance, respectively (Figure 2B). In both abdomen and head, all males from the four strains are separated from females along PC1. Notably, the two Y^M strains (that differ from each other by a single copy of a standard third chromosome) have the greatest separation of any pair of male samples along PC2 in the abdomen data. We observed similar results with a grade of membership model: males from all four strains show different membership from females in abdomen and head, and males from the

312 two different Y^M strains have the most different membership composition (Supplementary
313 Figure 3C). In head, one of the III^M genotypes is separated from the other males along PC2
314 (Figure 2B). In neither abdomen nor head do Y^M and III^M males have the greatest separation,
315 suggesting that the Y^M and III^M chromosomes affect gene expression to a similar extent as a non-
316 *Mdmd*-bearing, standard third chromosome.
317
318 We also identified individual genes with significant differential expression between strains using
319 DESeq2 (Love *et al.* 2014). Previous work found that an excess of third chromosome genes is
320 differentially expressed between Y^M and III^M males (Meisel *et al.* 2015), as expected based on
321 the differences in their genotypes. We similarly find that excesses of genes on the third
322 chromosome are differentially expressed in 5/8 comparisons between males with different III^M
323 chromosomes, between Y^M males that differ by a standard third chromosome, and between Y^M
324 and III^M males (Supplementary Figure 4). Only one other chromosome has a significant excess
325 of differentially expressed genes in a single comparison (chromosome II in the bwbCS-IsoCS
326 comparison). This amounts to one significant excess out of 32 total tests involving the other
327 major chromosomes (chromosomes I, II, IV, and V are involved in 4 pair-wise comparisons from
328 two tissue samples), which is less than the expected false-positive rate with a p-value cutoff of
329 0.05. Therefore, there is an excess of third chromosome genes with differential expression
330 between males that differ in their third chromosome genotype, regardless of whether the third
331 chromosome is a proto-Y (i.e., III^M) or autosomal.
332
333 There are more differentially expressed genes across the entire genome in the pair-wise
334 comparison between Y^M males with different standard third chromosomes than in any other pair-
335 wise comparison between males, including between Y^M and III^M males (Figure 2C-D,
336 Supplementary Figure 5, Supplementary Table 3). There are also similar fractions of
337 differentially expressed (sex-biased) genes when comparing each of the male genotypes with
338 females (Supplementary Figure 6). This provides further evidence that the III^M chromosome has
339 a minor effect on male gene expression, less than or equal to an autosomal third chromosome. In
340 summary, the PCA, grade of membership, and differential expression analyses all provide
341 consistent evidence that the III^M chromosome has a minor effect on global gene expression.
342
343 **Expression of genes in the house fly sex determination pathway following *Md-tra* knock**
344 **down**
345
346 To further examine the effect of the III^M chromosome on gene expression, we used RNAi
347 targeting *Md-tra* to create sex-reversed males that have a male phenotype and female genotype
348 without any male-determining proto-Y chromosome. The *Md-tra*-RNAi treatment causes sex
349 reversal by mimicking the effect of the male-determining *Mdmd* gene, which disrupts the
350 splicing of *Md-tra* and the positive autoregulatory function of *Md-tra* in the early embryo
351 (Hediger *et al.* 2010). We compared gene expression in the sex-reversed males with genotypic
352 males carrying a III^M chromosome. Our 2×2 experimental design consisted of injecting dsRNA
353 targeting either *Md-tra* (to sex-reverse genotypic females) or GFP (sham treatment) into
354 genotypic males and females (Table 1). We studied III^M males in this experiment because we can
355 use a morphological marker on the third chromosome to identify sex-reversed males (Hediger *et*
356 *al.* 2010).
357

358 We confirmed that *Md-tra*-RNAi knockdown does indeed result in reduced expression of *Md-tra*
359 in the sex-reversed male abdomens (Supplementary Figure 7A). In contrast, there is not a
360 significant difference in *Md-tra* expression between sex-reversed male heads and either normal
361 male or normal female heads (Supplementary Figure 7B). We discuss this observation further in
362 Supplementary Figure 7. We observe sex-reversal in the expression of *Md-dsx* and *Md-fru*, the
363 two immediate downstream targets of *Md-tra* (Hediger *et al.* 2004; Hediger *et al.* 2010; Meier *et*
364 *al.* 2013), in *Md-tra*-RNAi treated genotypic females (Supplementary Figure 7C-F).
365
366 **Expression profiles of sex-reversed males are similar to genotypic males, not phenotypic**
367 **females**
368
369 We next examined how the III^M chromosome affects gene expression in males using a PCA
370 analysis, grade of membership model, and hierarchical clustering to analyze RNA-seq data from
371 the four genotype-by-RNAi-treatment combinations (Table 1). In abdomen, PC1 explains 85%
372 of the variance in gene expression levels across samples. PC1 clusters all types of phenotypic
373 males together, including the sex-reversed males, separately from normal females (Figure 3A).
374 We observe a similar result in a grade of membership model and hierarchical clustering—the
375 sex-reversed males have similar abdominal expression profiles as genotypic males and different
376 from normal females (Supplementary Figures 8A and 9A). Therefore, the global gene expression
377 profile in abdomen is predicted by phenotypic sex (regulated by the sex determination pathway),
378 and not by sex chromosome genotype. This is consistent with the III^M chromosome having a
379 minor effect on male gene expression.
380
381 In head, we found that one of the sex-reversed males had elevated *Md-tra* expression
382 (Supplementary Figure 10A), suggesting incomplete knock down of *Md-tra*. In addition, the
383 RNA-seq profile of that sex-reversed male revealed it to be an outlier that did not cluster with
384 either normal females or genotypic males in a PCA (Supplementary Figure 10B). The PCA
385 showed that more variance in head gene expression can be explained by differences between that
386 outlier sex-reversed male and all other phenotypic males (including the other two sex-reversed
387 replicates) than between phenotypic males and females (Supplementary Figure 10B). We
388 therefore excluded that outlier sample because it is not consistent with sex-reversal.
389
390 After excluding the one sample, PC1 and PC2 explain 34% and 19% of the variance in gene
391 expression in head, respectively. PC1 for the head expression data separates normal females and
392 GFP-RNAi treated genotypic males (Figure 3B), providing more evidence that phenotypic sex is
393 a better predictor of overall gene expression profile than sex chromosome genotype. Curiously,
394 *Md-tra*-RNAi treated phenotypic males (which includes both sex-reversed males and III^M males
395 #2) were intermediate between GFP-RNAi treated normal females and males along head PC1,
396 and the *Md-tra*-RNAi flies were separated from GFP-RNAi treated flies along head PC2 (Figure
397 3B). The *Md-tra*-RNAi treated phenotypic male heads (sex-reversed males and III^M males #2)
398 also had reduced expression of *Md-fru* (Supplementary Figure 7F). We confirmed the similarity
399 between *Md-tra*-RNAi treated flies, regardless of genotypic sex, in both a grade of membership
400 model and via hierarchical clustering (Supplementary Figures 8B and 9B). Therefore, *Md-tra*
401 knock down appears to influence overall gene expression—as well as *Md-fru* expression or
402 splicing—in heads more than the effect of sex chromosome genotype.
403

404
405 **Sex-reversed and genotypic males have similar sex-biased gene expression**
406
407 Sexual dimorphism is achieved through differential (sex-biased) gene expression between males
408 and females (Ellegren and Parsch 2007). We compared sex-biased expression in sex-reversed
409 and genotypic males, excluding the one outlier sex-reversed male head. We used genotypic
410 males treated with GFP-RNAi (III^M males #1) as our normal male reference because the model
411 in DESeq2 we used for RNA-seq analysis allows for pair-wise comparisons between individuals
412 with either the same genotypic sex or treatment. Normal females and III^M males #1 both were
413 exposed to the GFP-RNAi treatment, which allows us to make the pairwise comparison (Table
414 1).
415
416 We first quantified the degree of sex-biased expression by the distribution of the $\log_2$ fold-
417 change between male and female expression levels ($\log_2$M/F). In the abdominal samples, the
418 distributions of $\log_2$M/F for sex-reversed and genotypic males, when compared to normal
419 females, are quite similar (Supplementary Figure 11A). We defined genes with sex-biased
420 expression as those with a $\log_2$M/F significantly different from 0 using DESeq2 (Love *et al.*
421 2014). Similar fractions of genes have sex-biased expression in abdomen for sex-reversed and
422 genotypic males: 11,005/14,686 (74.9%) of genes are significantly sex-biased in the comparison
423 between sex-reversed males and females, and 11,030/14,993 (73.6%) of genes have sex-biased
424 expression when comparing genotypic males and females (Figure 3C; Supplementary Table 4).
425 The distributions of $\log_2$M/F are symmetrical, with similar fractions of genes with male- and
426 female-biased expression for both sex-reversed and genotypic males (Supplementary Figure
427 11A). In contrast, the magnitude of differential gene expression is much smaller in comparisons
428 between genotypic males than male-female comparisons (Figure 3C; Supplementary Figure
429 11A). Notably, the proportion of differentially expressed genes is similar between sex-reversed
430 and genotypic males as between the two types of genotypic males (*Md-tra*-RNAi and GFP-RNAi
431 treated), providing additional evidence that sex-reversed males have similar gene expression
432 profiles as normal (genotypic) males (Figure 3C).
433
434 Sex-biased expression in fly heads is reduced relative to whole fly or gonad tissue (Goldman and
435 Arbeitman 2007; Lebo *et al.* 2009; Meisel *et al.* 2012, 2015). In house fly heads, we only detect
436 5,077 sex-biased genes between genotypic males and normal females out of 13,558 expressed
437 genes (Figure 3D; Supplementary Figure 11B; Supplementary Table 4). Similarly, there are only
438 735 sex-biased genes between sex-reversed males and normal females out of 12,360 expressed
439 genes (Figure 3D; Supplementary Figure 11B; Supplementary Table 4). The lower number of
440 sex-biased genes between sex-reversed males and normal females could be a result of decreased
441 power because of a smaller sample size; only two replicate sex-reversed male heads were
442 included because the third replicate had an outlier expression profile (see above). The
443 alternative—that sex-reversed male heads are less sexual dimorphic than genotypic male
444 heads—is unlikely because sex-reversed male head gene expression profiles are most similar to
445 genotypic males in both a PCA and (Figure 3B; Supplementary Figure 9B). In addition, there are
446 fewer genes differentially expressed in head between sex-reversed males and *Md-tra*-RNAi
447 treated genotypic males (III^M males #2) than between the two types of genotypic males (Figure
448 3D). This result is consistent with the grouping of sex-reversed male and *Md-tra*-RNAi treated
449 genotypic male head gene expression profiles in both a PCA and hierarchical clustering (Figure

450 3B; Supplementary Figure 9B), suggesting that gene expression in head is more affected by *Md-*
451 *tra*-RNAi than by the III^M chromosome.
452
453 We next tested if the same genes have sex-biased expression in sex-reversed males and
454 genotypic males (III^M males #1). In both abdomen and head, the majority of male-biased genes
455 in genotypic males are also male-biased in sex-reversed males (Figure 3E-F). The same is true
456 for female-biased genes. We assessed if the sex-biased genes in common between sex-reversed
457 and genotypic males is greater than expected by chance with a permutation test. We determined a
458 null distribution assuming that sex-biased genes in the sex-reversed and genotypic males are
459 independent of each other from 1,000 random permutations of our data. The actual number of
460 male- and female-biased genes in common between sex-reversed males and genotypic males is
461 much greater than all values in the null distribution (Supplementary Figure 12). This result
462 implies that sexual dimorphism is achieved by similar means in both sex-reversed males and
463 genotypic males: silencing of *Md-tra*, independent of alleles on the III^M chromosome.
464
465 **Disproportionate differential expression of third chromosome genes**
466
467 Although all phenotypic males, regardless of genotypic sex, showed very similar gene expression
468 profiles, we identified some genes that are differentially expressed between genotypic males and
469 sex-reversed males (Figure 3). These differentially expressed genes could reveal important
470 phenotypic effects of the III^M proto-Y chromosome, which may be targets for selection to
471 maintain both Y^M and III^M across house fly populations. We therefore further examined
472 differential expression between sex-reversed and genotypic males to determine the effect of the
473 III^M chromosome on individual genes. As expected based on their genotypic differences, there
474 are significant excesses of third chromosome genes differentially expressed between genotypic
475 III^M males and sex-reversed males in abdomen and head (Figure 4). There is also a significant
476 excess of third chromosome genes differentially expressed between genotypic males and normal
477 females in head (Figure 4B). In contrast, there is not an excess of third chromosome genes
478 differentially expressed between normal females and sex-reversed males (Figure 4), who share
479 the same genotype. These patterns are consistent with our previous work (Meisel *et al.* 2015) and
480 other results presented here (Supplementary Figure 4) in which the third chromosome has an
481 excess of differentially expressed genes between flies that differ in their third chromosome
482 genotype. However, we surprisingly find that there are excesses of differentially expressed genes
483 on the third chromosome in comparisons between III^M males with the *Md-tra*-RNAi and GFP-
484 RNAi treatments (Figure 4). Therefore, in addition to the expected genotypic effects, dsRNA
485 targeting *Md-tra* and/or GFP disproportionately affects the expression of genes on the house fly
486 third chromosome.
487
488 Sex reversed males and genotypic males have very similar expression profiles (Figure 3). In spite
489 of these similarities, we identified some "discordant sex-biased genes" that have sex-biased
490 expression in either sex-reversed or genotypic males, but not both (Figure 3E-F). To further
491 examine the effect of the III^M chromosome on gene expression, we divided the discordant sex-
492 biased genes into two groups: "sex-reversed-up-discordant" and "normal-up-discordant". We
493 considered a gene to be sex-reversed-up-discordant if it belongs to one of two categories: 1)
494 male-biased expression in sex-reversed males and not male-biased in genotypic males
495 ($\log_2 M/F<0$ but not necessarily significant), or 2) female-biased expression in genotypic males

496 and not female-biased in sex-reversed males ($log_2M/F>0$ but not necessarily significant). These
497 genes are pooled into a single group because they have higher expression in sex-reversed males.
498 We identified 49 sex-reversed-up-discordant genes in abdomens and 170 in heads (Figure 5A;
499 Supplementary Table 5; Supplementary Data). Likewise, we classified genes as normal-up-
500 discordant if they are in one of two categories: 1) male-biased expression in genotypic (normal)
501 males and not male-biased in sex-reversed males ($log_2M/F<0$ but not necessarily significant), or
502 2) female-biased expression in sex-reversed males and not female-biased expression in normal
503 males ($log_2M/F>0$ but not necessarily significant). These genes are pooled together because they
504 have higher expression in normal males. We identified 25 normal-up-discordant genes in
505 abdomens and 418 in heads (Figure 5B; Supplementary Table 5; Supplementary Data). There are
506 no GO terms significantly enriched in either the sex-reversed-up-discordant or normal-up-
507 discordant genes. However, both sex-reversed-up-discordant and normal-up-discordant genes in
508 both abdomen and head are significantly enriched on the third chromosome (Figure 5;
509 Supplementary Tables 6 and 7). Therefore, in comparisons between males with and without a
510 III^M chromosome, the third chromosome is enriched for genes with discordant sex-biased
511 expression.
512
513
514 **Discussion**
515
516 The house fly Y^M and III^M proto-Y chromosomes are geographically distributed in a way that
517 suggests ecological factors favor different proto-Y chromosomes across different habitats
518 (Franco *et al.* 1982; Tomita and Wada 1989; Hamm *et al.* 2005; Feldmeyer *et al.* 2008;
519 Kozielska *et al.* 2008). This predicts that there will be sequence differences between the proto-Y
520 chromosomes and their homologous (proto-X) chromosomes that confer ecologically dependent
521 phenotypic and fitness effects. These differences could be in transcribed sequences (e.g., protein
522 coding genes) or in regulatory regions that control their expression. Paradoxically, however, both
523 the Y^M and III^M chromosomes have minimal sequence differences relative to their homologous
524 chromosomes (Meisel *et al.* 2017).
525
526 We tested if minimal sequence differences between the Y^M and III^M proto-Y chromosomes and
527 their homologs could be responsible for phenotypic effects by investigating gene expression
528 differences between males carrying different proto-Y chromosomes. We hypothesize that gene
529 expression differences between males with different proto-Y chromosomes are caused by
530 differences between proto-Y chromosomes and their homologs (e.g., III^M and the standard third
531 chromosome) in their *cis*-regulatory sequences, the *trans*-regulatory factors they encode, and any
532 downstream effects. The *cis*-regulatory differences should disproportionately affect expression of
533 genes on the proto-Y chromosomes. Consistent with that prediction, there is a significant excess
534 of genes that are differentially expressed on the third chromosome when comparing males with
535 and without the III^M proto-Y (Figure 4; Supplementary Figure 4), as previously described
536 (Meisel et al. 2015).
537
538 We compared gene expression in four house fly strains carrying either a Y^M or III^M chromosome
539 on a common genetic background (Figure 1). The nearly isogenic strains were created by
540 backcrossing the proto-Y chromosomes onto the common background (e.g., Supplementary
541 Figure 1). Male recombination is absent in most flies (Gethman 1988), suggesting that our strains

542 contain intact proto-Y chromosomes inherited from the progenitor strain. However, there is some
543 evidence for male recombination in house flies (Feldmeyer et al. 2010). If recombination
544 occurred between the proto-Y chromosomes and their homologs during backcrossing, then
545 portions of the proto-sex chromosomes would also be isogenic across the strains we examined.
546 Regardless of the extent of male recombination, expression differences between the strains
547 should be (almost) entirely the result of genetic differences in proto-Y chromosome sequences
548 (III^M or Y^M) between strains, even if the entire proto-Y chromosome was not introgressed.
549 Notably, the biggest differences in gene expression were observed between two Y^M strains
550 carrying different standard third chromosomes (not carrying *Mdmd*), and not between III^M and
551 Y^M males (Figure 2). Our results therefore suggest that the magnitude of gene expression
552 differences between III^M and Y^M males can be explained by replacing a chromosome on a
553 common genetic background, and they are not specific to the effect of the III^M or Y^M
554 chromosomes.
555
556 We also examined the effects of the III^M chromosome on male gene expression using an RNAi
557 experiment (Table 1). We chose to knock down *Md-tra* because it allows us to create sex-
558 reversed fertile males that do not carry any proto-Y chromosomes, as opposed to knock
559 down/out of *Mdmd*, which creates sex-reversed fertile females carrying a proto-Y (Sharma *et al.*
560 2017). We found that gene expression profiles of sex-reversed and normal (genotypic) males are
561 very similar—phenotypic male expression profiles are distinct from phenotypic females,
562 regardless of male genotype (Figures 3). We therefore conclude that the III^M chromosome has a
563 minor effect on male gene expression in a constant environment as assayed in our experiments. It
564 is worth noting that we only considered virgin females in our experiments, and mating can affect
565 female gene expression in flies (e.g., McGraw et al. 2004; Dalton et al. 2010). However, it is
566 unlikely that including mated females would have dramatically affected our central finding—
567 mated females should have global gene expression profiles that are more similar to virgin
568 females than phenotypic males.
569
570 Despite the minimal effect of the proto-Y chromosomes on global gene expression, we identified
571 74 genes in abdomen and 588 genes in head with sex-biased expression that is affected by the
572 III^M chromosome. These genes with discordant sex-biased expression are enriched on the third
573 chromosome (Figure 5). Discordant sex-biased genes could be targets of selection involved in
574 the maintenance of polygenic sex determination. The small number of genes whose expression is
575 affected by the proto-Y chromosomes suggests that the selection pressures responsible for
576 maintaining the proto-Y chromosome polymorphism likely act on a limited number of genetic
577 targets, not the entirety of the proto-Y chromosomes.
578
579 **Gene expression effects of the proto-Y chromosomes**
580
581 A previous experiment identified many genes whose expression differs between Y^M and III^M
582 males (Meisel *et al.* 2015). That experiment controlled for genetic background, but it did not
583 compare the effect of the proto-Y chromosomes with the effects of equivalent autosomes. When
584 we included the effect of introducing an autosome onto the same genetic background, we
585 observe more expression differences between Y^M males that carry different copies of standard
586 (non-*Mdmd*-bearing or autosomal) third chromosomes than between Y^M males and III^M males
587 (Figure 2). This minimal effect of the III^M proto-Y chromosome on expression, relative to a

588 standard third chromosome, suggests that III^M has very few differences from the standard third
589 chromosome, and III^M is not a fully "masculinized" Y chromosome (Rice 1996). In addition, III^M
590 likely is not differentiated enough from the standard third chromosome to require dosage
591 compensation in heterogametic males. Alternatively, III^M males may have a dosage
592 compensation mechanism (i.e., through preferred expression of genes on the standard third
593 chromosomes), which could mask the effects of the III^M chromosome on gene expression.
594 Additional work is necessary to compare these hypotheses.
595
596 It is curious that the Y^M males with different standard third chromosomes have more expression
597 differences than between Y^M and III^M males (Figure 2). One explanation for the amount of
598 expression differences between the Y^M males is that the standard third chromosome in our
599 experiment has a greater effect on gene expression than the III^M chromosome. Alternatively, the
600 different origins of the Y^M chromosomes in the two Y^M genotypes could have a large effect on
601 gene expression. Unfortunately, our experimental design prevents us from differentiating
602 between the effects of the Y^M chromosomes and standard third chromosome on the expression
603 differences between these Y^M males. However, if differences between Y^M chromosomes were
604 responsible for the elevated differential expression between the two Y^M male genotypes, this
605 would suggest that variation amongst the effects of Y^M chromosomes in our experiment exceeds
606 differences between Y^M and X chromosomes. Non-recombining Y chromosomes are expected to
607 have low levels of polymorphism (Clark 1987, 1988). However, variation across
608 *D. melanogaster* Y chromosomes has been shown to affect gene expression across the genome
609 and may be involved in the resolution of sexual conflicts (Lemos *et al.* 2008, 2010). In addition,
610 human Y chromosomes harbor high levels of copy number variation of ampliconic genes (Ye *et*
611 *al.* 2018). Intriguingly, the house fly Y^M chromosome carries recently duplicated genes that
612 differentiate it from the homologous X chromosome (Meisel *et al.* 2017). If these Y^M
613 duplications vary in their copy number or if there are chromatin-level differences across Y^M
614 chromosomes, this could explain a possible effect of the Y^M chromosome on global gene
615 expression. Additional work is necessary to test these hypotheses.
616
617 **The III^M chromosome, *cis*-regulation, and sexual conflicts**
618
619 Despite the minimal effects of the III^M chromosome on global gene expression profiles, we do
620 identify two notable patterns across all types of males. First, higher proportions of genes on the
621 third chromosome, relative to other chromosomes, are differentially expressed in many of our
622 comparisons between males with different genotypes (Figure 4; Supplementary Figure 4). This
623 might be indicative of divergence of *cis*-regulatory alleles between the III^M and standard third
624 chromosomes. The differentially expressed third chromosome genes could have important
625 phenotypic effects that could partially be responsible for fitness differences between males with
626 and without the III^M chromosome. Those fitness differences could in turn explain the
627 maintenance of both the Y^M and III^M proto-Y chromosomes in natural populations. Additional
628 work is necessary to connect individual differentially expressed genes to fitness effects of the
629 III^M chromosome.
630
631 Second, genes with discordant sex-biased expression between genotypic males and sex-reversed
632 males are over-represented on the third chromosome (Figure 5). This is consistent with our
633 previous results showing that the III^M chromosome disproportionately promotes male-biased

634 expression (Meisel *et al.* 2015). These results are contingent on inference of the chromosomal
635 assignment of house fly genes, which we have confirmed is accurate by comparing with an
636 independent mapping approach (Meisel and Scott 2018).
637
638 Population genetics theory predicts that sexually antagonistic selection is a major driver of the
639 evolution of sex determination and the maintenance of polygenic sex determination (Orzack *et*
640 *al.* 1980; van Doorn and Kirkpatrick 2007, 2010; Meisel *et al.* 2016). For example, sexual
641 conflicts could be resolved if sexually antagonistic alleles are inherited in a sex-limited manner
642 through the origination of a tightly linked sex-determining factor (Lindholm and Breden 2002;
643 Roberts *et al.* 2009; Ser *et al.* 2010; Parnell *et al*. 2013). In addition, male-beneficial alleles are
644 expected to accumulate on proto-Y chromosomes once they have acquired male-limited
645 inheritance (Rice 1992). The excess of discordant sex-biased genes on the third chromosome
646 may be consistent with these theoretical predictions if the up- or down-regulation of these genes
647 on the III^M chromosome is beneficial to males and deleterious to females. In this case, the male-
648 beneficial (and female-detrimental) alleles would be *cis*-regulatory elements that affect the
649 expression of the discordant sex-biased genes on the III^M chromosome (Figure 5). A similar
650 phenomenon was observed in Lake Malawi cichlids, where an allele underlying a sexually
651 antagonistic pigmentation phenotype is a *cis*-regulatory variant that up-regulates the expression
652 of a gene linked to a new sex determiner (Roberts *et al.* 2009). Although the house fly male
653 determiner (*Mdmd*) is molecularly characterized (Sharma *et al.* 2017), its location on the III^M
654 chromosome is not known, which prevents us from testing if the discordant sex-biased genes are
655 nearby and genetically linked to the male determiner.
656
657 There are two considerations, however, that may be important limitations of these
658 interpretations. First, the fitness effects of the proto-Y chromosomes appear to be
659 environmentally dependent. Y^M is most frequent at northern latitudes and III^M predominates in
660 the south, suggesting that temperature-dependent fitness differences could be responsible for
661 north-south clines (Franco *et al.* 1982; Tomita and Wada 1989; Hamm *et al.* 2005; Feldmeyer *et*
662 *al.* 2008; Kozielska *et al.* 2008). We did not test for temperature-dependent effects of the proto-Y
663 chromosomes in our experiment, which may have prevented us from identifying key fitness-
664 related gene expression differences between Y^M and III^M males. These temperature-dependent
665 effects could be the result of temperature-dependent expression of genes on Y^M and III^M,
666 differences in temperature-dependent activity of the copies of *Mdmd* across proto-Y
667 chromosomes, or some other temperature-dependent genotype-by-environment interaction.
668 Second, Y^M and III^M can be carried by females who also carry the epistatic *Md-tra*^{D} allele
669 (McDonald *et al.* 1978; Hediger *et al.* 2010). The fitness differences between Y^M and III^M could
670 therefore be mediated through the effects of the proto-Y chromosomes on female phenotypes,
671 which we did not assay in our experiments. Additional work is necessary to investigate how
672 temperature and sex modulate the phenotypic effects of the proto-Y chromosomes.
673
674 **The effect of *Md-tra* on gene expression**
675
676 Our results suggest that *Md-tra* has effects on gene expression beyond the direct regulation of
677 *Md-dsx* and *Md-fru* splicing. Previous results, as well as our experiments here, demonstrate that
678 knock down of *Md-tra* in blastoderm embryos causes complete sex-reversal of genotypic females
679 into fertile phenotypic males (Hediger *et al.* 2010). Our results suggest that this sex-reversal does

not affect all adult tissues equally—we observed one fertile sex-reversed male with higher *Md-tra* expression than normal females in head and a head gene expression profile that does not cluster with phenotypic females or males (Supplementary Figure 10). Curiously, the outlier sex-reversed male in our experiment does not have a gene expression profile intermediate between genotypic males and females (Supplementary Figure 10B), as we would expect from partial masculinization. This suggests *Md-tra*-RNAi treatment in blastoderm embryo can have effects on adult somatic gene expression that does not act in the expected direction of sex-reversal.

We find additional evidence that *Md-tra* knockdown can affect adult gene expression independently of genotype. For example, the two genotypic males in our RNAi knockdown experiment have the same genotypic and phenotypic sex, yet their head gene expression profiles are not the most similar of all genotype-by-treatment combinations; instead, genotypic males with *Md-tra*-RNAi treatment cluster with sex-reversed males (Figure 3B; Supplementary Figure 9B). There are also more genes differentially expressed in head between III^M males with and without *Md-tra*-RNAi treatment than between genotypic males and sex-reversed males (Figure 3D). These results suggest that *Md-tra* affects head gene expression independent of genotypic sex. The effects of *Md-tra*-RNAi on head expression are likely mediated through either direct effects of *Md-tra* on the splicing of transcripts other than *Md-dsx* and *Md-fru*, downstream effects of *Md-dsx* and *Md-fru* alternative splicing, or off-target effects of dsRNA targeting *Md-tra*. In contrast, we do not observe a disproportionate effect of *Md-tra*-RNAi on abdominal gene expression—knocking down *Md-tra* converts genotypic females into phenotypic (sex-reversed) males with expression profiles that nearly perfectly mimic genotypic (normal) males (Figure 3A,C,E; Supplementary Figures 8A and 9A).

Notably, the expression of *Md-tra* does not differ across the heads of genotypic males or females with either RNAi treatment (Supplementary Figure 7B). This suggests that the expression effects of knocking down *Md-tra* in adult heads is not through direct effects on *Md-tra*, but instead is caused by off-target effects or downstream effects of the direct targets of *Md-tra*. It is therefore possible that silencing *Md-tra* in early blastoderm embryos affects regulatory pathways that modulate head gene expression independently of the activity *Md-tra* in adult heads. Sex determination in flies is cell autonomous, and many cells in *Drosophila* somatic tissues do not express sex-determining genes downstream of *tra* (Robinett *et al.* 2010). Our results suggest that even if somatic tissues do not differentially express sex-determining genes, they can carry the memory of regulation of the sex determination pathway from their progenitor cells.

Curiously, there is an excess of third chromosome genes differentially expressed between III^M males with *Md-tra*-RNAi treatment and III^M males with *GFP*-RNAi treatment (Figure 4). The III^M chromosome is a proto-Y, and the standard third chromosome is a proto-X. Therefore, knockdown of *Md-tra* could be disproportionately affecting proto-Y genes or proto-X genes. Unfortunately, our data lack the resolution to determine if the expression changes between III^M males with different RNAi treatments is the result of changes in expression of genes on the III^M chromosome, standard third chromosome, or both. Regardless of which homolog is changing in expression, one explanation for the disproportionate effect of *Md-tra* knockdown on third chromosome genes is that there is an excess of third chromosome targets regulated by *Md-tra* or the sex determination pathway. For example, the house fly sex determination pathway could regulate gene expression specifically on the proto-X chromosome, analogous to how *Drosophila*

X chromosome dosage compensation is controlled in a sex-specific manner by a gene in the sex determination pathway (Salz and Erickson 2010). Intriguingly, knockdown of *transformer* in female red flour beetles, *Tribolium castaneum*, causes them to produce nearly all male progeny, possibly as a result of misregulation of the diploid X chromosome in the female progeny (Shukla and Palli 2012). *Md-tra* in house fly may have a similar role regulating X chromosome expression. Additional work is necessary to evaluate why *Md-tra* knockdown disproportionately affects third chromosome expression.

Conclusions

We have performed multiple RNA-seq experiments to resolve the paradox of ecologically relevant fitness effects of the house fly Y^M and III^M proto-Y chromosomes despite minimal sequence divergence between proto-Y and proto-X chromosomes. We identified some effects of the Y^M and III^M chromosomes on gene expression, but the number of differentially expressed genes and their effect sizes are small relative to the effect of a standard third chromosome or knockdown of the key sex determining gene *Md-tra*. Therefore, gene expression in house flies depends more on phenotypic sex (mediated by the sex determination pathway) than sex chromosome genotype. Despite the minimal effect of house fly proto-Y chromosomes on global gene expression, there are individual genes on the III^M chromosome that are differentially expressed relative to the standard third chromosome. Those differentially expressed genes may represent targets of selection that is responsible for maintaining polygenic sex determination.

Our results are consistent with a recent study in *Rana temporaria* frogs that have polygenic sex determination, which found that sex-biased gene expression depends more on phenotypic sex than genotypic sex (Ma *et al.* 2018). Combining the findings from flies and frogs suggests that the earliest stage of sex chromosome evolution is dominated by changes in the expression of individual genes, rather than transcriptome-wide effects of the young sex chromosomes. In the case of house fly, it is possible that the individual gene expression differences could act in an ecologically-dependent manner. For example, the geographic distribution of the Y^M and III^M chromosomes could arise from selection on environmentally sensitive phenotypes that we did not assay in our experiments. Because seasonality of temperature is predictive of the frequencies of Y^M and III^M in natural populations (Feldmeyer *et al.* 2008), a fitness or phenotypic assay across temperatures may be needed to identify ecologically relevant differences between Y^M and III^M males.

Acknowledgements

This work was supported by a grant from the National Science Foundation (OISE 1444220) to RPM. We thank Christopher Gonzales for assistance with preparation of the RNA-seq libraries, and Claudia Brunner for technical assistance with the RNAi knockdown experiment. All RNA-seq data were generated at the University of Houston Seq-N-Edit core and analyzed on the Maxwell Cluster at the University of Houston Core facility for Advanced Computing and Data Science. We also thank Ernst Wimmer, Louis van de Zande, Elzemiek Geuverink, Martijn Schenkel, and Xuan Li for fruitful discussions on housefly sex determination evolution. Three anonymous reviewers provided valuable feedback that improved this manuscript.

773
774 **Tables**
775
776
777 **Table 1.** Injection scheme for RNAi treatments in both sexes.
778

Genotypic Sex	RNAi treatment	
	GFP-RNAi	*Md-tra*-RNAi
Genotypic Female (III/III)	(A) Phenotypic Female (III/III) "females"	(B) Phenotypic Male (III/III) "sex-reversed males"
Genotypic Male (III^M/III)	(C) Phenotypic Male (III^M/III) "III^M males #1"	(D) Phenotypic Male (III^M/III) "III^M males #2"

779
780 Genotypic females with the *Md-tra*-RNAi treatment are sex-reversed to phenotypic males (**B**).
781 The other genotypic females and males are not sex-reversed (**A, C, D**); their phenotypic sexes
782 are congruent with their genotypic sexes (i.e., normal males or females).
783

References Cited

Anders S., P. T. Pyl, and W. Huber, 2015 HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics 31: 166–169.

Bachtrog D., J. E. Mank, C. L. Peichel, M. Kirkpatrick, S. P. Otto, *et al.*, 2014 Sex determination: why so many ways of doing it? PLoS Biol. 12: e1001899.

Benjamini Y., and Y. Hochberg, 1995 Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Series B Stat. Methodol. 57: 289–300.

Beukeboom L. W., and N. Perrin, 2014 *The Evolution of Sex Determination*. Oxford University Press.

Boyes J. W., M. J. Corey, and H. E. Paterson, 1964 Somatic chromosomes of higher diptera: IX. Karyotypes of some muscid species. Can. J. Zool. 42: 1025–1036.

Clark A. G., 1987 Natural selection and Y-linked polymorphism. Genetics 115: 569–577.

Clark A. G., 1988 The evolution of the Y chromosome with X-Y recombination. Genetics 119: 711–720.

Dalton, J.E., Kacheria, T.S., Knott, S.R., Lebo, M.S., Nishitani, A., Sanders, L.E., Stirling, E.J., Winbush, A. and Arbeitman, M.N., 2010 Dynamic, mating-induced gene expression changes in female head and brain tissues of Drosophila melanogaster. BMC Genomics 11: 541.

Dey K. K., C. J. Hsiao, and M. Stephens, 2017 Visualizing the structure of RNA-seq expression data using grade of membership models. PLoS Genet. 13: e1006599.

Doorn G. S. van, and M. Kirkpatrick, 2007 Turnover of sex chromosomes induced by sexual conflict. Nature 449: 909–912.

Doorn G. S. van, and M. Kirkpatrick, 2010 Transitions between male and female heterogamety caused by sex-antagonistic selection. Genetics 186: 629–645.

805 Dübendorfer A., M. Hediger, G. Burghardt, and D. Bopp, 2002 *Musca domestica*, a window on the
806 evolution of sex-determining mechanisms in insects. Int. J. Dev. Biol. 46: 75–79.

807 Ellegren H., and J. Parsch, 2007 The evolution of sex-biased genes and sex-biased gene expression. Nat.
808 Rev. Genet. 8: 689–698.

809 Feldmeyer B., M. Kozielska, B. Kuijper, F. J. Weissing, L. W. Beukeboom, *et al.*, 2008 Climatic
810 variation and the geographical distribution of sex-determining mechanisms in the housefly. Evol.
811 Ecol. Res. 10: 797–809.

812 Feldmeyer, B., Pen, I. and Beukeboom, L.W., 2010 A microsatellite marker linkage map of the housefly,
813 *Musca domestica*: evidence for male recombination. Insect Mol Biol. 19: 575–581.

814 Foster G. G., M. J. Whitten, C. Konovalov, J. T. A. Arnold, and G. Maffi, 1981 Autosomal genetic maps
815 of the Australian Sheep Blowfly, *Lucilia cuprina dorsalis* R.-D. (Diptera: Calliphoridae), and
816 possible correlations with the linkage maps of *Musca domestica* L. and *Drosophila melanogaster*
817 (Mg.). Genet. Res. 37: 55–69.

818 Franco M. G., P. G. Rubini, and M. Vecchi, 1982 Sex-determinants and their distribution in various
819 populations of Musca domestica L. of Western Europe. Genet. Res. 40: 279–293.

820 Gethmann, R.C., 1988 Crossing over in males of higher Diptera (Brachycera). J. Hered, 79: 344–350.

821 Goldman T. D., and M. N. Arbeitman, 2007 Genomic and functional studies of Drosophila sex hierarchy
822 regulated gene expression in adult head and nervous system tissues. PLoS Genet. 3: e216.

823 Hamm R. L., T. Shono, and J. G. Scott, 2005 A cline in frequency of autosomal males is not associated
824 with insecticide resistance in house fly (Diptera: Muscidae). J. Econ. Entomol. 98: 171–176.

825 Hamm R. L., J.-R. Gao, G. G.-H. Lin, and J. G. Scott, 2009 Selective advantage for III^M males over Y^M
826 males in cage competition, mating competition, and pupal emergence in *Musca domestica* L.

827 (Diptera: Muscidae). Environ. Entomol. 38: 499–504.

828 Hamm R. L., R. P. Meisel, and J. G. Scott, 2015 The evolving puzzle of autosomal versus Y-linked male
829 determination in Musca domestica. G3 5: 371–384.

830 Hediger M., A. D. Minet, M. Niessen, R. Schmidt, D. Hilfiker-Kleiner, *et al.*, 1998 The male-determining
831 activity on the Y chromosome of the housefly (*Musca domestica* L.) consists of separable elements.
832 Genetics 150: 651–661.

833 Hediger M., M. Niessen, E. A. Wimmer, A. Dübendorfer, and D. Bopp, 2001 Genetic transformation of
834 the housefly *Musca domestica* with the lepidopteran derived transposon *piggyBac*. Insect Mol. Biol.
835 10: 113–119.

836 Hediger M., G. Burghardt, C. Siegenthaler, N. Buser, D. Hilfiker-Kleiner, *et al.*, 2004 Sex determination
837 in *Drosophila melanogaster* and *Musca domestica* converges at the level of the terminal regulator
838 *doublesex*. Dev. Genes Evol. 214: 29–42.

839 Hediger M., C. Henggeler, N. Meier, R. Perez, G. Saccone, *et al.*, 2010 Molecular characterization of the
840 key switch *F* provides a basis for understanding the rapid divergence of the sex-determining pathway
841 in the housefly. Genetics 184: 155–170.

842 Huang D. W., B. T. Sherman, and R. A. Lempicki, 2009 Systematic and integrative analysis of large gene
843 lists using DAVID bioinformatics resources. Nat. Protoc. 4: 44–57.

844 Kim D., B. Langmead, and S. L. Salzberg, 2015 HISAT: a fast spliced aligner with low memory
845 requirements. Nat. Methods 12: 357–360.

846 Kolde, R., 2019 pheatmap: Pretty Heatmaps. R package version 1.0.12.
847 https://CRAN.R-project.org/package=pheatmap

848 Kozielska M., I. Pen, L. W. Beukeboom, and F. J. Weissing, 2006 Sex ratio selection and multi-factorial

849 sex determination in the housefly: a dynamic model. J. Evol. Biol. 19: 879–888.

850 Kozielska M., B. Feldmeyer, I. Pen, F. J. Weissing, and L. W. Beukeboom, 2008 Are autosomal sex-
851 determining factors of the housefly (Musca domestica) spreading north? Genet. Res. 90: 157–165.

852 Lebo M. S., L. E. Sanders, F. Sun, and M. N. Arbeitman, 2009 Somatic, germline and sex hierarchy
853 regulated gene expression during Drosophila metamorphosis. BMC Genomics 10: 80.

854 Lemos B., L. O. Araripe, and D. L. Hartl, 2008 Polymorphic Y chromosomes harbor cryptic variation
855 with manifold functional consequences. Science 319: 91–93.

856 Lemos B., A. T. Branco, and D. L. Hartl, 2010 Epigenetic effects of polymorphic Y chromosomes
857 modulate chromatin components, immune response, and sexual conflict. Proc. Natl. Acad. Sci. U. S.
858 A. 107: 15826–15831.

859 Li H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, *et al.*, 2009 The Sequence Alignment/Map format
860 and SAMtools. Bioinformatics 25: 2078–2079.

861 Lindholm, A. and Breden, F., 2002 Sex chromosomes and sexual selection in poeciliid fishes.
862 Am. Nat. 160: 214–224.

863 Love M. I., W. Huber, and S. Anders, 2014 Moderated estimation of fold change and dispersion for
864 RNA-seq data with DESeq2. Genome Biol. 15: 550.

865 Ma W.-J., P. Veltsos, R. Sermier, D. J. Parker, and N. Perrin, 2018 Evolutionary and developmental
866 dynamics of sex-biased gene expression in common frogs with proto-Y chromosomes. Genome Biol.
867 19: 156.

868 Mcdonald I. c., P. Evenson, C. A. Nickel, and O. A. Johnson, 1978 House Fly Genetics: Isolation of a
869 Female Determining Factor on Chromosome 4. Ann. Entomol. Soc. Am. 71: 692–694.

870 McGraw, L.A., Gibson, G., Clark, A.G. and Wolfner, M.F., 2004 Genes regulated by mating, sperm, or
871 seminal proteins in mated female *Drosophila melanogaster*. Curr. Biol. 14:.1509-1514.

872 Meier N., S. C. Käppeli, M. Hediger Niessen, J.-C. Billeter, S. F. Goodwin, *et al.*, 2013 Genetic control of
873 courtship behavior in the housefly: evidence for a conserved bifurcation of the sex-determining
874 pathway. PLoS One 8: e62476.

875 Meisel R. P., J. H. Malone, and A. G. Clark, 2012 Disentangling the relationship between sex-biased gene
876 expression and X-linkage. Genome Res. 22: 1255–1265.

877 Meisel R. P., J. G. Scott, and A. G. Clark, 2015 Transcriptome Differences between Alternative Sex
878 Determining Genotypes in the House Fly, Musca domestica. Genome Biol. Evol. 7: 2051–2061.

879 Meisel R. P., T. Davey, J. H. Son, A. C. Gerry, T. Shono, *et al.*, 2016 Is Multifactorial Sex Determination
880 in the House Fly, Musca domestica (L.), Stable Over Time? J. Hered. 107: 615–625.

881 Meisel R. P., C. A. Gonzales, and H. Luu, 2017 The house fly Y Chromosome is young and minimally
882 differentiated from its ancient X Chromosome partner. Genome Res. 27: 1417–1426.

883 Meisel R. P., and J. G. Scott, 2018 Using genomic data to study insecticide resistance in the house fly,
884 Musca domestica. Pestic. Biochem. Physiol. 151: 76–81.

885 Moore E. C., and R. B. Roberts, 2013 Polygenic sex determination. Curr. Biol. 23: R510–2.

886 Orzack S. H., J. J. Sohn, K. D. Kallman, S. A. Levin, and R. Johnston, 1980 Maintenance of the three sex
887 chromosome polymorphism in the platyfish *Xiphophorus maculatus*. Evolution 34: 663–672.

888 Parnell N. F., and J. T. Streelman, 2013 Genetic interactions controlling sex and color establish the
889 potential for sexual conflict in Lake Malawi cichlid fishes. Heredity 110: 239–246.

890 Rice W. R., 1986 On the Instability of Polygenic Sex Determination: The Effect of Sex- Specific

891 Selection. Evolution 40: 633–639.

892 Rice W. R., 1992 Sexually antagonistic genes: experimental evidence. Science 256: 1436–1439.

893 Rice W. R., 1996 Evolution of the Y Sex Chromosome in Animals. Bioscience 46: 331–343.

894 Roberts R. B., J. R. Ser, and T. D. Kocher, 2009 Sexual conflict resolved by invasion of a novel sex
895 determiner in Lake Malawi cichlid fishes. Science 326: 998–1001.

896 Robinett C. C., A. G. Vaughan, J.-M. Knapp, and B. S. Baker, 2010 Sex and the single cell. II. There is a
897 time and place for sex. PLoS Biol. 8: e1000365.

898 Salz H. K., and J. W. Erickson, 2010 Sex determination in Drosophila: The view from the top. Fly 4: 60–
899 70.

900 Schmidt R., M. Hediger, R. Nöthiger, and A. Dübendorfer, 1997 The mutation *masculinizer* (*man*)
901 defines a sex-determining gene with maternal and zygotic functions in *Musca domestica* L. Genetics
902 145: 173–183.

903 Scott J. G., P. Sridhar, and N. Liu, 1996 Adult specific expression and induction of cytochrome P450lpr
904 in house flies. Arch. Insect Biochem. Physiol. 31: 313–323.

905 Scott J. G., W. C. Warren, L. W. Beukeboom, D. Bopp, A. G. Clark, *et al.*, 2014 Genome of the house
906 fly, *Musca domestica* L., a global vector of diseases with adaptations to a septic environment.
907 Genome Biol. 15: 466.

908 Ser J. R., R. B. Roberts, and T. D. Kocher, 2010 Multiple interacting loci control sex determination in
909 lake Malawi cichlid fish. Evolution 64: 486–501.

910 Sharma A., S. D. Heinze, Y. Wu, T. Kohlbrenner, I. Morilla, *et al.*, 2017 Male sex in houseflies is
911 determined by Mdmd, a paralog of the generic splice factor gene CWC22. Science 356: 642–645.

912 Shono T., and J. G. Scott, 2003 Spinosad resistance in the housefly, Musca domestica, is due to a
913 recessive factor on autosome 1. Pestic. Biochem. Physiol. 75: 1–7.

914 Shukla J. N., and S. R. Palli, 2012 Sex determination in beetles: production of all male progeny by
915 parental RNAi knockdown of transformer. Sci. Rep. 2: 602.

916 Tomita T., and Y. Wada, 1989 Multifactorial sex determination in natural populations of the housefly
917 (Musca domestica) in Japan. The Japanese Journal of Genetics 64: 373–382.

918 Vicoso B., and D. Bachtrog, 2013 Reversal of an ancient sex chromosome to an autosome in Drosophila.
919 Nature 499: 332–335.

920 Weller G. L., and G. G. Foster, 1993 Genetic maps of the sheep blowfly *Lucilia cuprina*: linkage-group
921 correlations with other dipteran genera. Genome 36: 495–506.

922 Ye D., A. A. Zaidi, M. Tomaszkiewicz, K. Anthony, C. Liebowitz, *et al.*, 2018 High Levels of Copy
923 Number Variation of Ampliconic Genes across Major Human Y Haplogroups. Genome Biol. Evol.
924 10: 1333–1350.

925

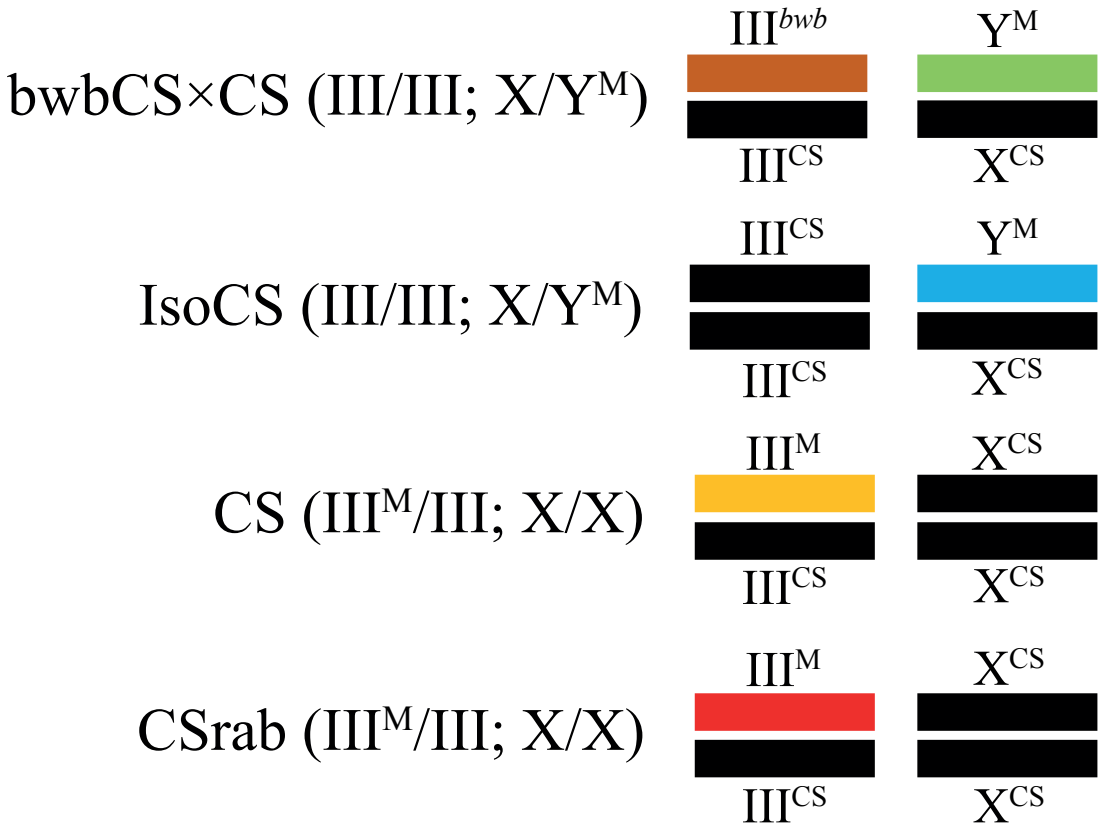


Figure 1. The genotypes of four strains with different naturally occurring Y^M or III^M proto-Y chromosomes on a common genetic background are shown. Black bars represent chromosomes from the common genetic background, and colored bars are chromosomes that are replaced on that background. Different colors of chromosomes indicate their origins from different strains. Chromosomes in the rest of genome (not shown), are from the common genetic background as well.

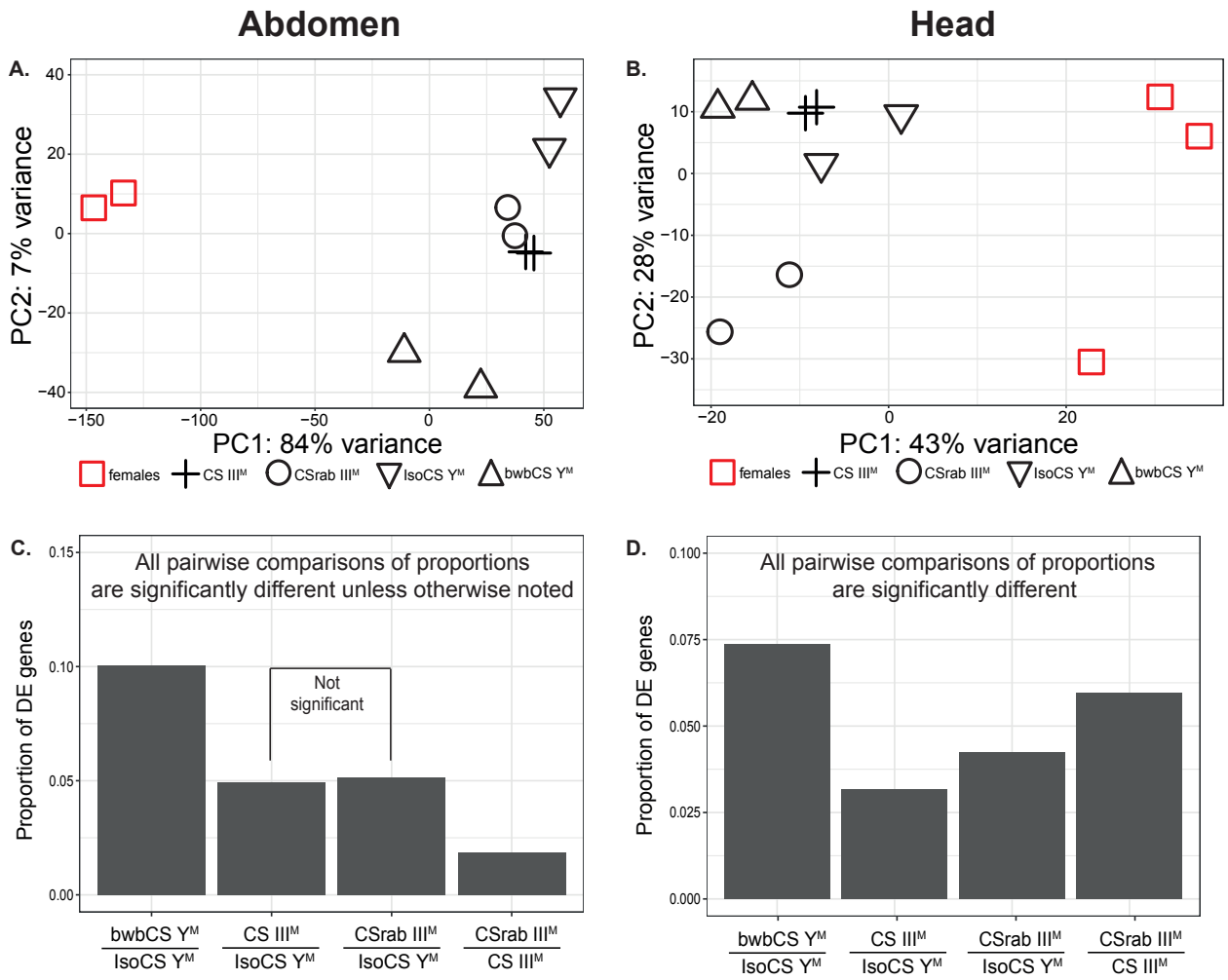


Figure 2. Plots show the results of a PCA of global expression in males with different proto-Y chromosomes in abdomen (**A**) and head (**B**). Bar graphs show the proportions of differentially expressed (DE) genes between males with different proto-Y chromosomes in abdomens (**C**) and heads (**D**). bwbCS Y^M stands for the strain bwbCS×CS.

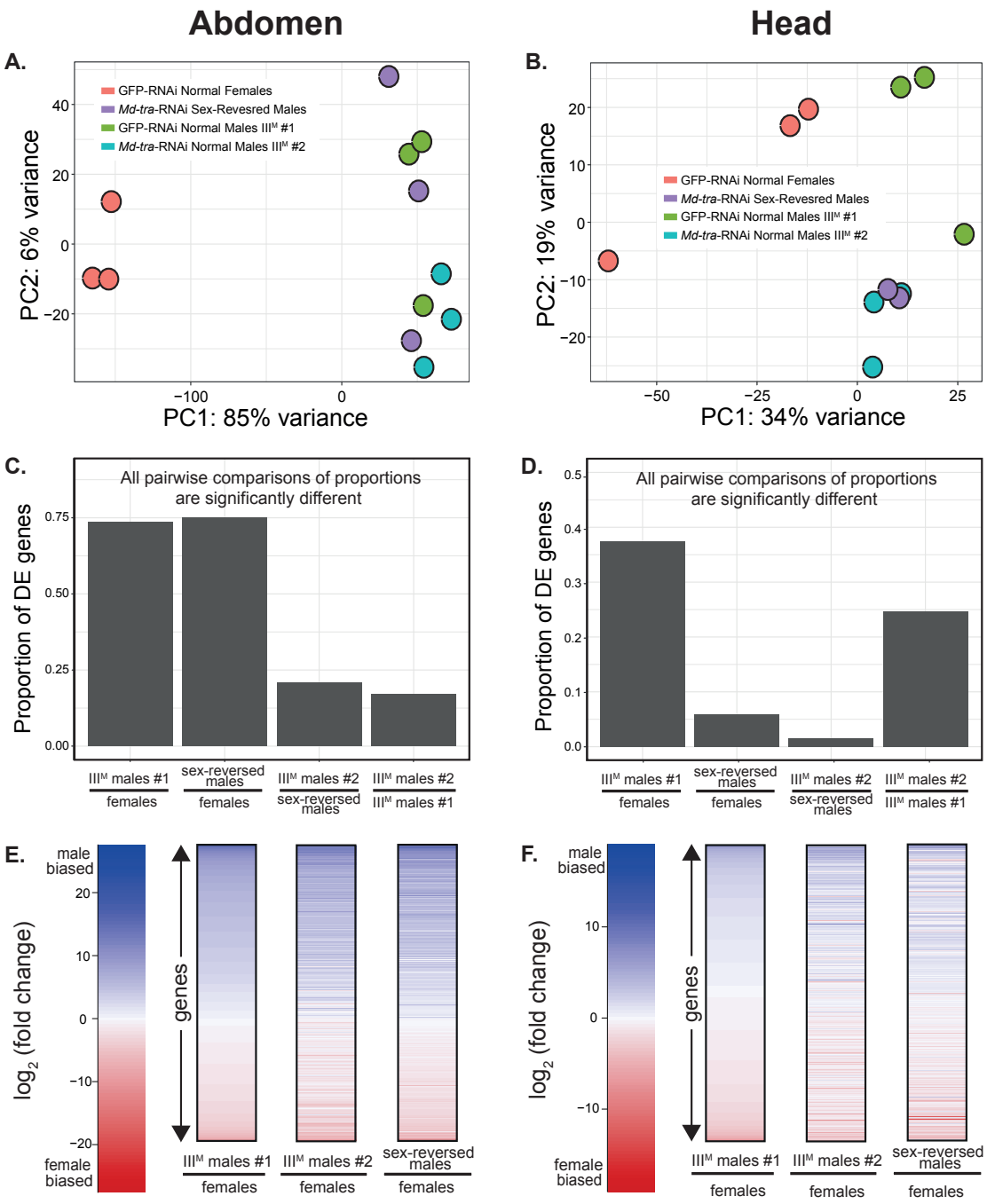


Figure 3. Plots show the results of a PCA of global expression of GFP-RNAi and *Md-tra*-RNAi treated genotypic females and males in abdomen (**A**) and head (**B**). Bar graphs show the proportions of differentially expressed (DE) genes between different types of individuals in abdomen (**C**) and head (**D**). "Females" refers to GFP-RNAi treated normal females. Heat maps show expression differences between each type of male and phenotypic females in abdomen (**E**) and head (**F**). Genes are ordered the same in all three comparisons within each tissue, such that a row in comparison contains the same gene as the corresponding row in the other two comparisons.

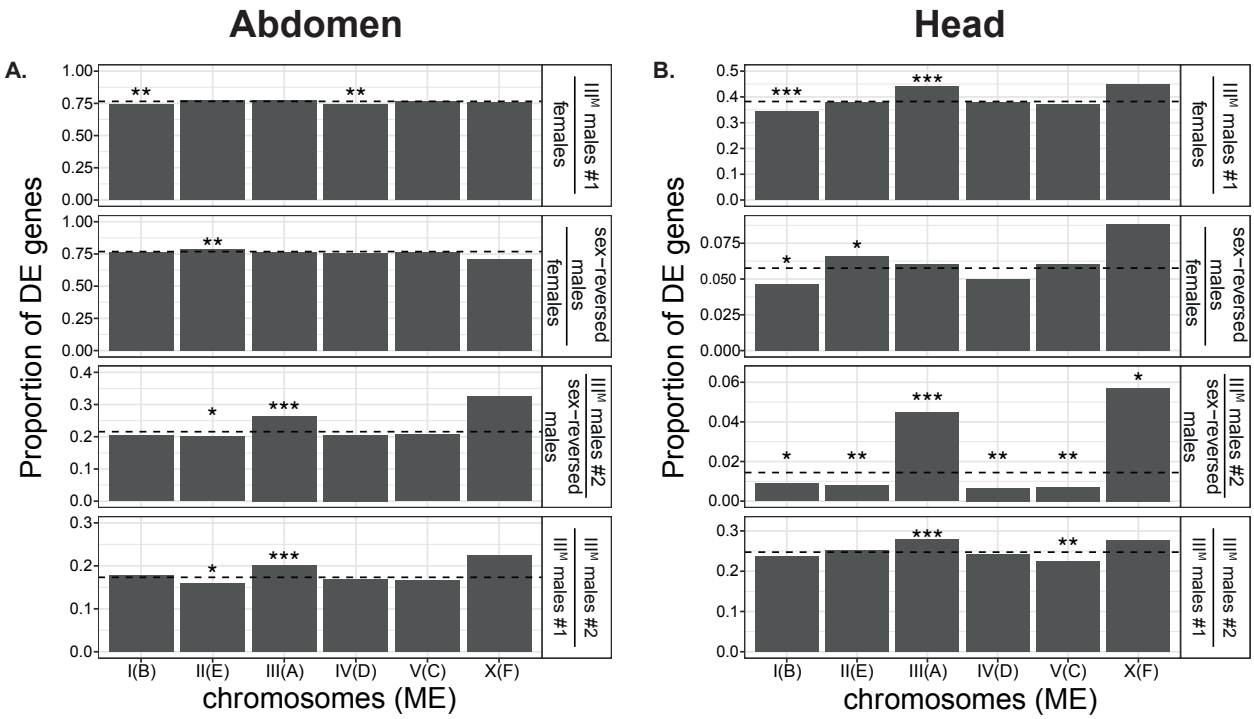


Figure 4. Bar graphs show the proportions of genes on each chromosome (*Drosophila* Muller element in parentheses) that are differentially expressed (DE) between genotype and treatment combinations in abdomen (**A**) and head (**B**). Asterisks indicate significant differences based on Fisher's exact test (**P*<0.05, ***P*<0.01, ****P* < 0.001).

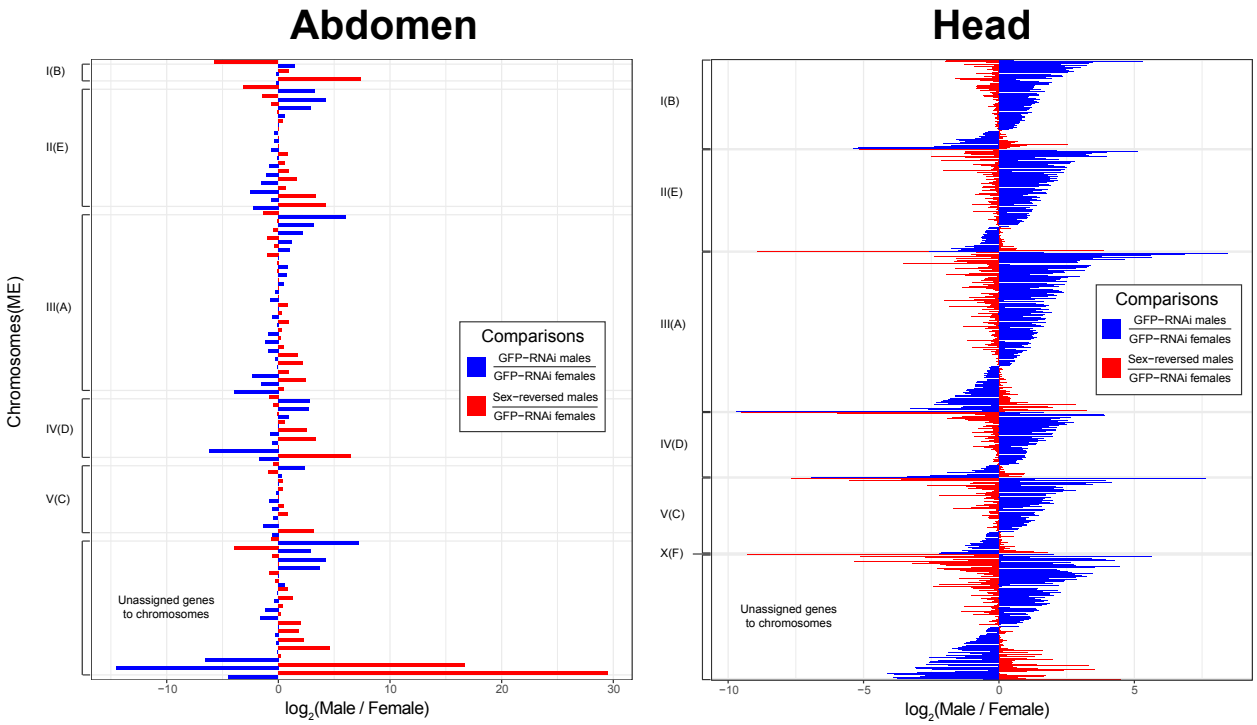
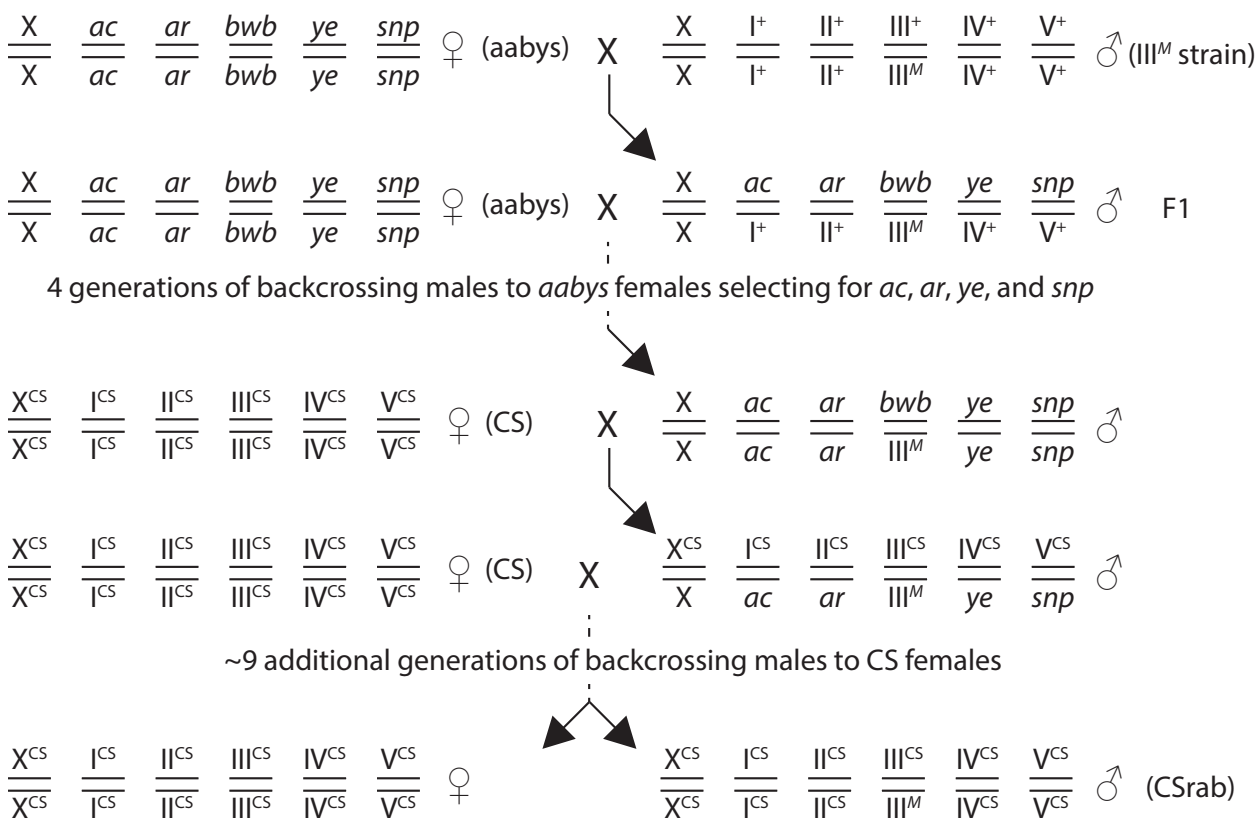
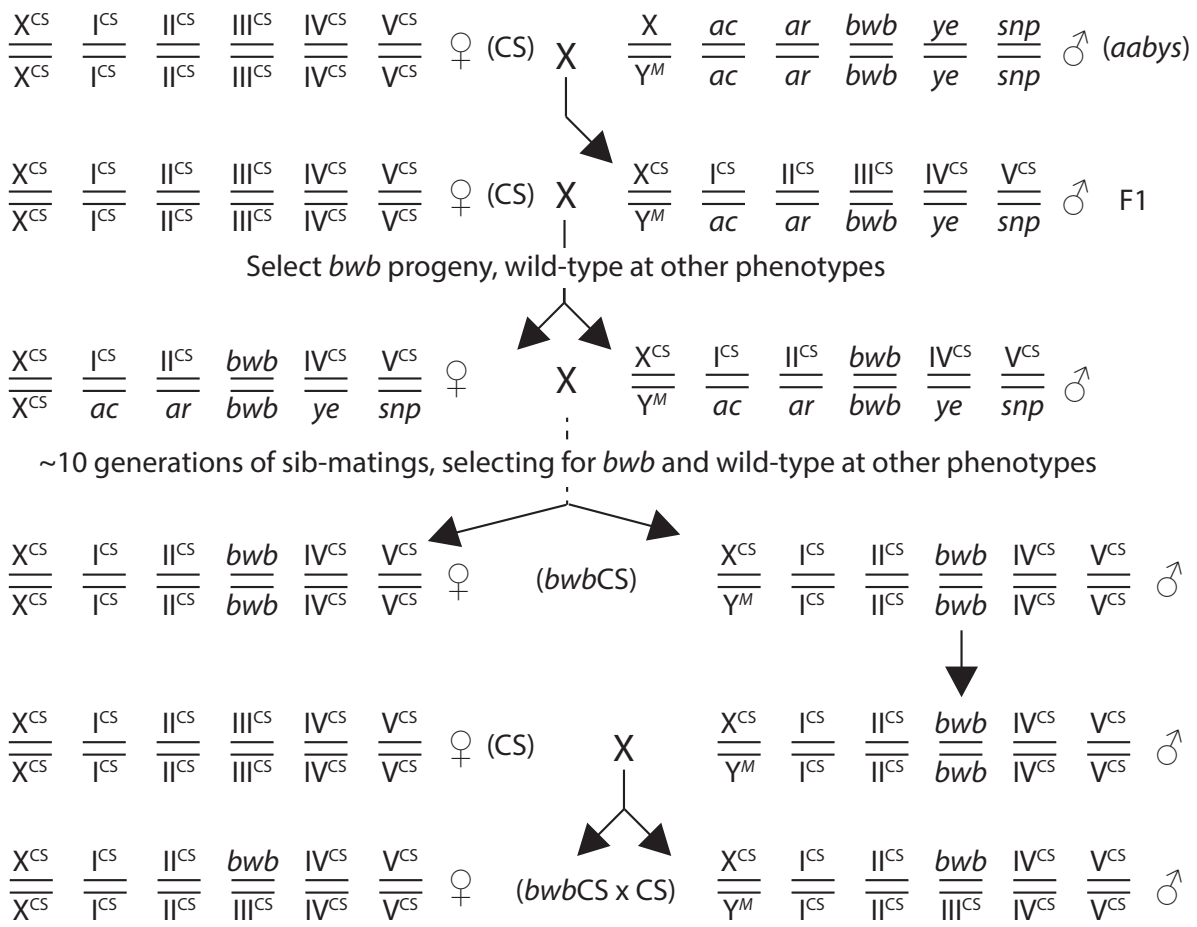


Figure 5. Chromosomal locations of discordant sex-biased genes in abdomen (**A**) and head (**B**) are shown. Blue bars indicate the degree of sex-biased expression ($\log_2$M/F) in genotypic males (III^M males #1) compared to females, and red bars show sex-biased expression in sex-reversed males compared to females. Each row represents a single gene with discordant sex-biased expression, with one blue bar and one red bar per gene.

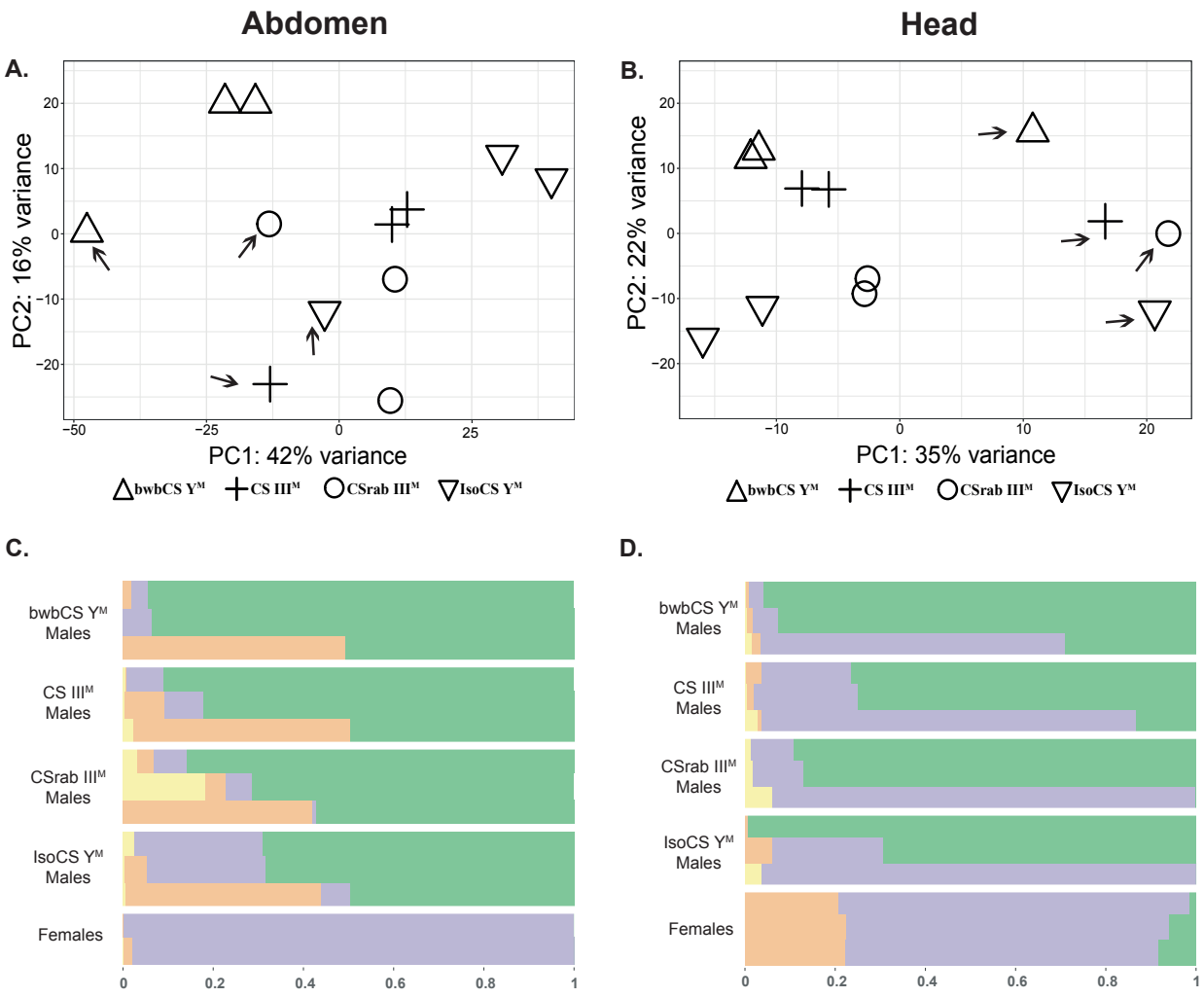
Supplementary Material



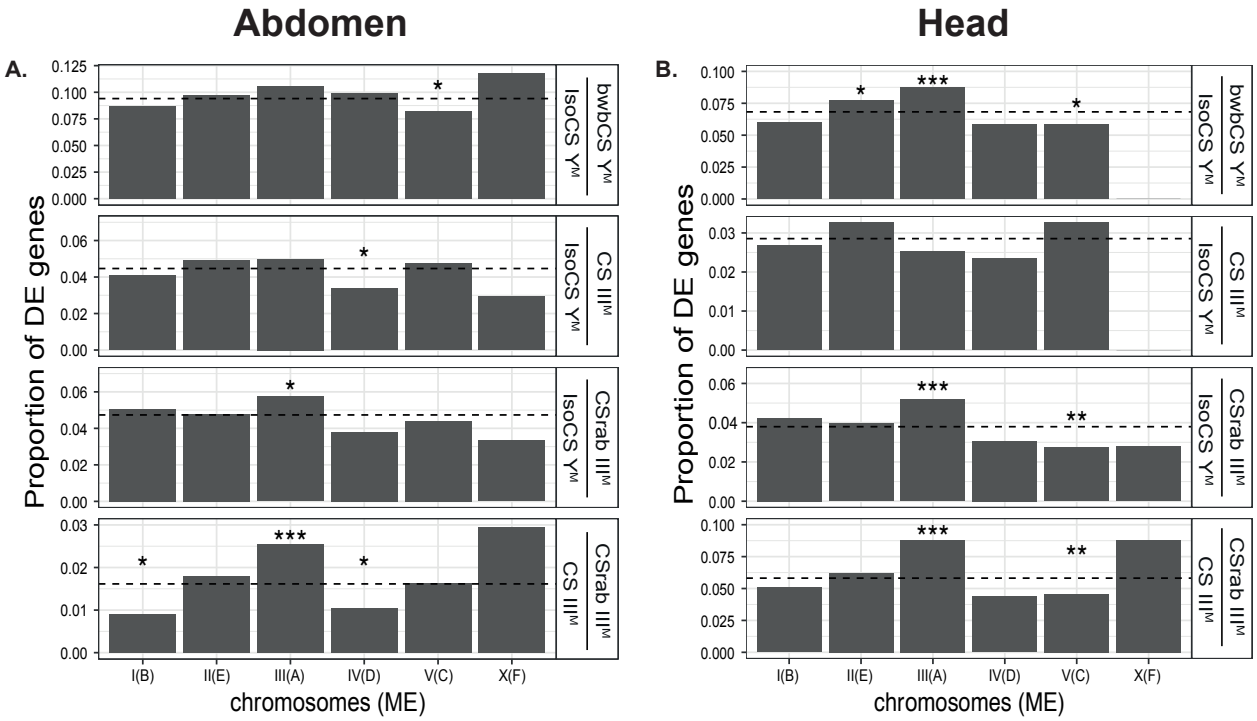
Supplementary Figure 1. Crossing scheme used to create the CSrab strain. Each pair of parallel horizonal bars represents homologous chromosomes; there are six pairs of chromosomes (X/Y, I, II, III, IV, V). The aabys strain has a recessive phenotypic marker on each autosome.



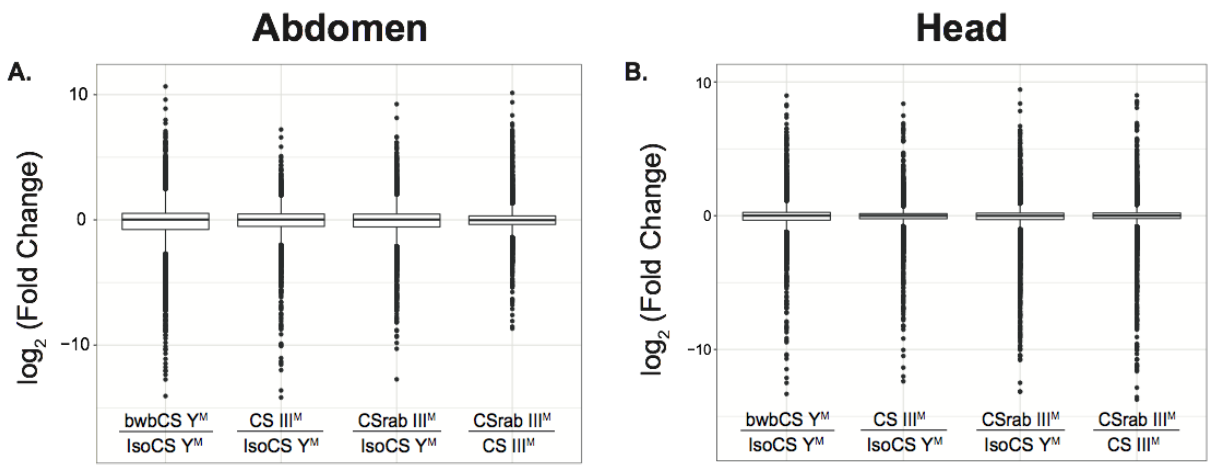
Supplementary Figure 2. Crossing scheme to create the *bwb*CS strain and *bwb*CS × CS line used in this experiment. Each pair of parallel horizonal bars represents homologous chromosomes; there are six pairs of chromosomes (X/Y, I, II, III, IV, V). The aabys strain has a recessive phenotypic marker on each autosome.



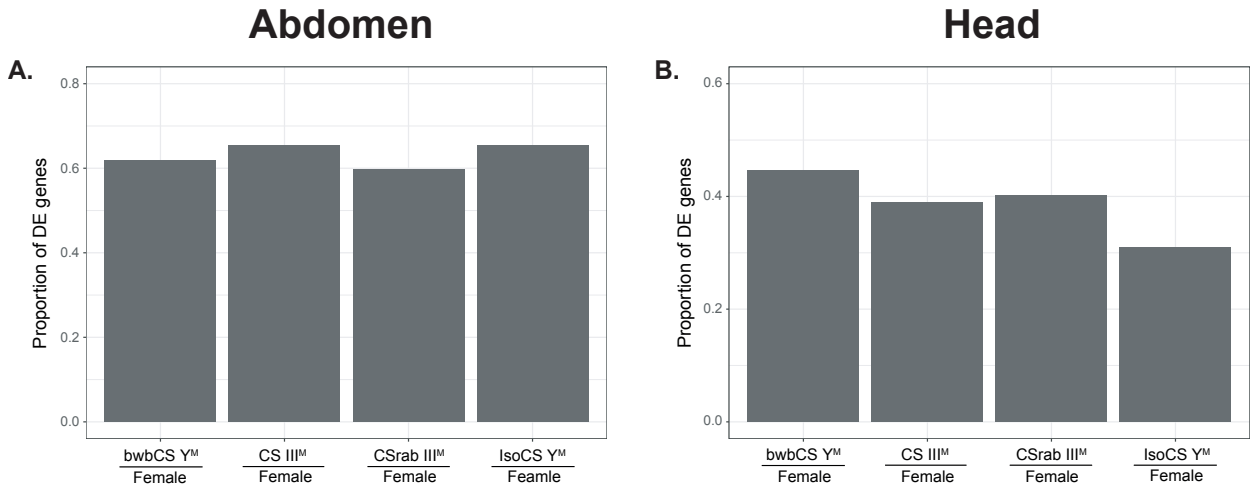
Supplementary Figure 3. (**A, B**) Principal component (PC) analysis of four strains that have different naturally occurring proto-Y chromosomes on a common genetic background in abdomens (**A**) and heads (**B**). Arrows point to outlier samples, one for each of the four strains. Female abdomens are excluded from the PCA (**A**) to show the outliers. (**C, D**) Grade of membership model ($K = 4$) for gene expression patterns of four strains that have different naturally occurring proto-Y chromosomes on a common genetic background in abdomens (**C**) and heads (**D**). Each row represents one replicate of a genotype, with the outliers excluded. Each color represents the proportion of each replicate assigned to each of the three clusters.



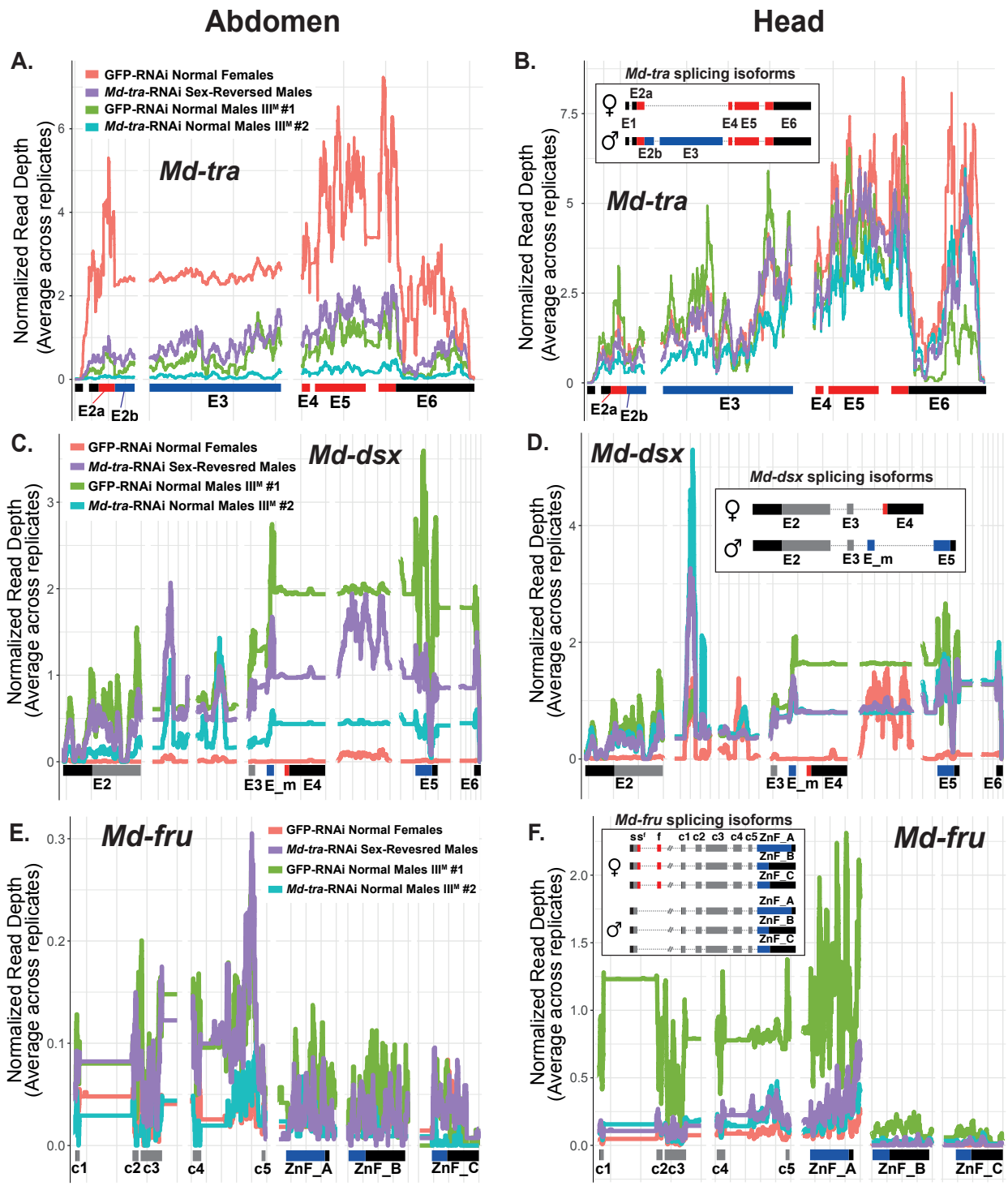
Supplementary Figure 4. Bar graphs indicate the proportions of genes on each chromosome (*Drosophila* Muller element in parentheses) that are differentially expressed (DE) between different male genotypes in abdomen (**A**) and head (**B**). Asterisks indicate significant differences based on Fisher's exact test comparing the number of DE genes on a chromosome against the number of DE genes in the rest of the genome (**P*<0.05, ***P*<0.01, ****P* < 0.001).



Supplementary Figure 5. Boxplots show fold changes of gene expression between males with different *Mdmd*-bearing chromosomes in abdomens (**A**) and heads (**B**). Outliers are included as points.



Supplementary Figure 6. Bar graphs show the proportions of differentially expressed (DE) genes between females and males with different Y^M or III^M proto-Y chromosomes in abdomens (**A**) and heads (**B**). bwbCS Y^M stands for the strain bwbCS×CS.



Supplementary Figure 7. The graphs show read depth coverage of *Md-tra* (**A, B**), *Md-dsx* (**C, D**) and *Md-fru* (**E, F**) in abdomens (**A, C, E**) and heads (**B, D, F**) of flies with different RNAi treatments. Exons of *Md-tra*, *Md-dsx*, and *Md-fru* are presented along the X-axis. The names of the *Md-tra*, *Md-dsx*, and *Md-fru* exons follow published nomenclature (Hediger *et al.* 2004, 2010; Meier *et al.* 2013). Insets show female and male isoforms of *Md-tra*, *Md-dsx* and *Md-fru*, respectively. In *Md-tra*, Blue exons (E2b, E3) that contain premature stop codons are included in

the male isoforms of *Md-tra* but excluded from the female isoforms. In *Md-fru*, red exons (s^f and f) that are contained in female isoforms have premature stop codons, but are excluded from the male isoforms. Exons (s,s^f,f) upstream from an exon 'c1' of *Md-fru* are not included in the read depth coverage because they are not on the same scaffold in the genome assembly.

To confirm that the *Md-tra*-RNAi treatment knocks down *Md-tra* expression, we examined the expression of *Md-tra* using RNA-seq coverage data collected from the abdomen and head of each of our four sample types (**A, B**). We expect the expression of *Md-tra* in females to be higher than in males because males produce a splice variant with a premature stop codon that is likely to be processed by the nonsense-mediated decay (NMD) pathway (Hediger *et al.* 2010; Kervestin and Jacobson 2012). In addition, the ovaries are expected to produce large amounts of *Md-tra* transcripts because *Md-tra* activity is necessary for maternal establishment of zygotic splicing of *Md-tra* via an auto-regulatory loop (Dübendorfer and Hediger 1998). In abdomen, normal females (GFP-RNAi treated genotypic females) do indeed express *Md-tra* approximately three times higher than normal males (genotypic males with either the GFP-RNAi or *Md-tra*-RNAi treatment). This high *Md-tra* expression in female abdomens might reflect the outcomes of strong ovarian expression. Importantly, *Md-tra* expression in sex-reversed males (genotypic females that are phenotypic males because of *Md-tra*-RNAi) was comparable to that of the genotypic males, not the normal females (**A**). This is likely because knock down of *Md-tra* by RNAi produces sex-reversed males that have functioning testes instead of ovaries. The *Md-tra* exons that are included in the functional, female-determining transcript were the highest expressed exons in phenotypic females (**A**), consistent with the production of the female-determining transcript in female ovaries (Hediger *et al.* 2010).

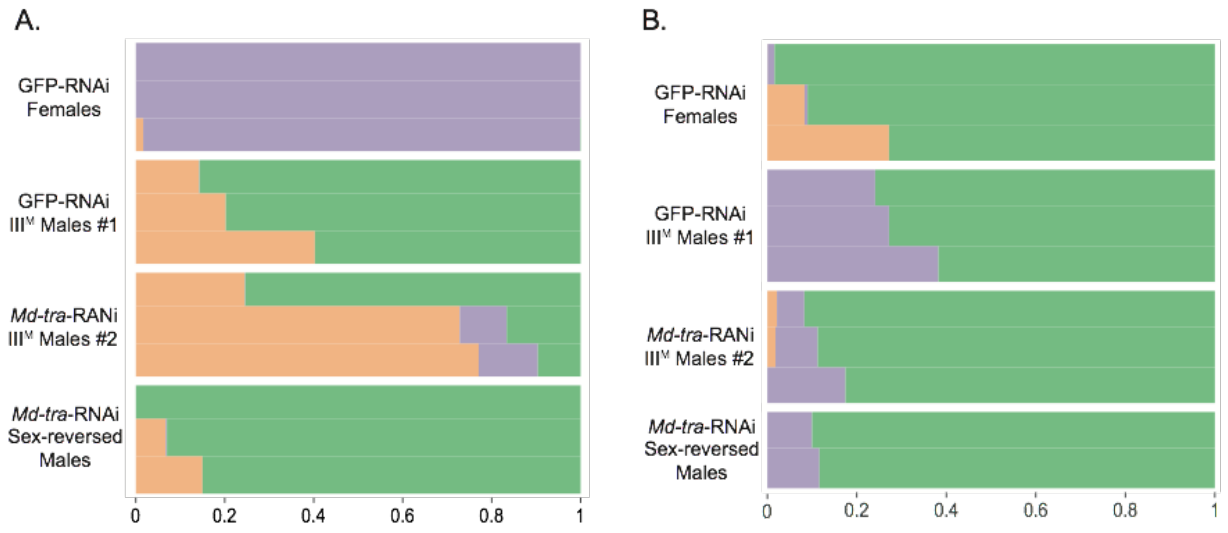
We find that *Md-tra* is also differentially expressed between females and males in head, but the difference is much smaller than in abdomen (**B**). Notably, when we analyze the read mapping to *Md-tra* using DESeq2, expression is significantly higher in normal females than in genotypic (III^M #1) males. However, there is not a significant difference in *Md-tra* expression between sex-reversed males and either normal males or normal females. These results were observed after we excluded a sex-reversed male head sample that had an outlier expression profile (see Main Text). The lack of sexually dimorphic expression of *Md-tra* in head is consistent with minimal sex-biased expression in *Drosophila* and house fly heads (Goldman and Arbeitman 2007; Meisel *et al.* 2015). In addition, most somatic cells in *Drosophila* are sexually monomorphic as a result of cell autonomous sex determination in *Drosophila* (Robinett *et al.* 2010), suggesting that the same may be true for most cells in house fly heads.

Md-tra regulates the splicing of at least two downstream genes, *Md-dsx* and *Md-fru*, which are both differentially spliced between females and males (Hediger *et al.* 2004, 2010; Meier *et al.* 2013). Only the female isoform of *Md-tra* is translated into a functional protein. In the presence of Md-Tra, *Md-dsx* is spliced into an isoform that promotes female morphological development. *Md-dsx* is spliced into an isoform that initiates male morphological development in the absence of Md-Tra (Hediger *et al.* 2004, 2010). *Md-fru* is spliced into a male-specific behavioral regulator in the absence of Md-Tra (Meier *et al.* 2013).

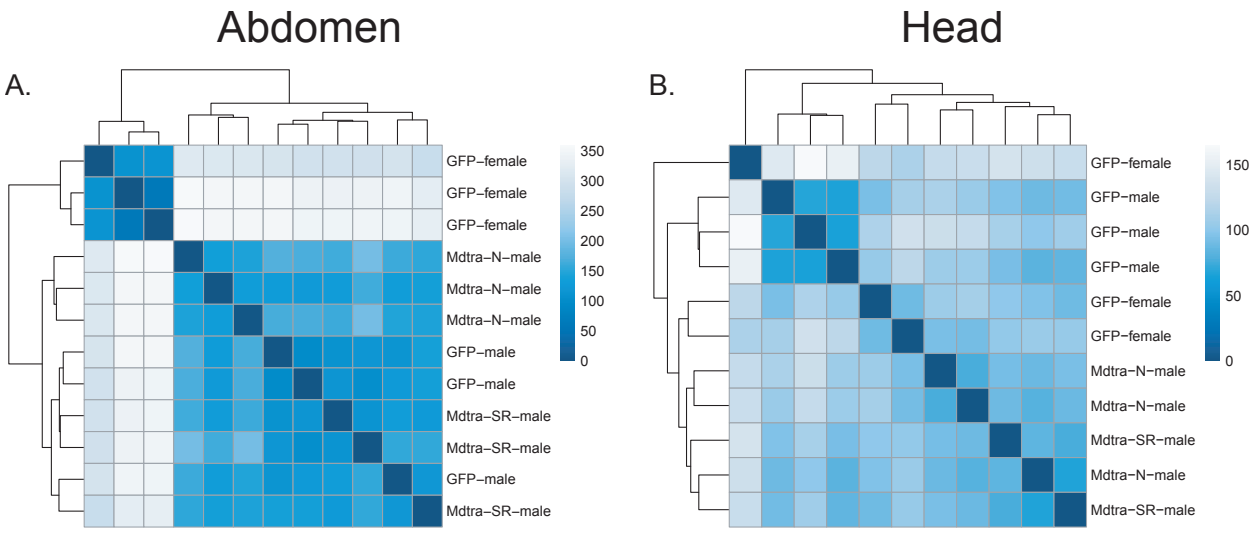
The expression of *Md-dsx* and *Md-fru* in sex-reversed males was more similar to that of normal (genotypic) males (especially *Md-tra*-RNAi treated III^M males #2) than phenotypic females (**C-**

F), confirming that *Md-tra* knock down affects the downstream genes in the sex determination pathway (Hediger *et al.* 2010; Meier *et al.* 2013). For example, *Md-dsx* expression in phenotypic males was higher than in phenotypic females, especially across male-specific exons (**C-D**), consistent with the expected effect of Md-TRA on *Md-dsx* splicing in females (Hediger *et al.* 2004, 2010).

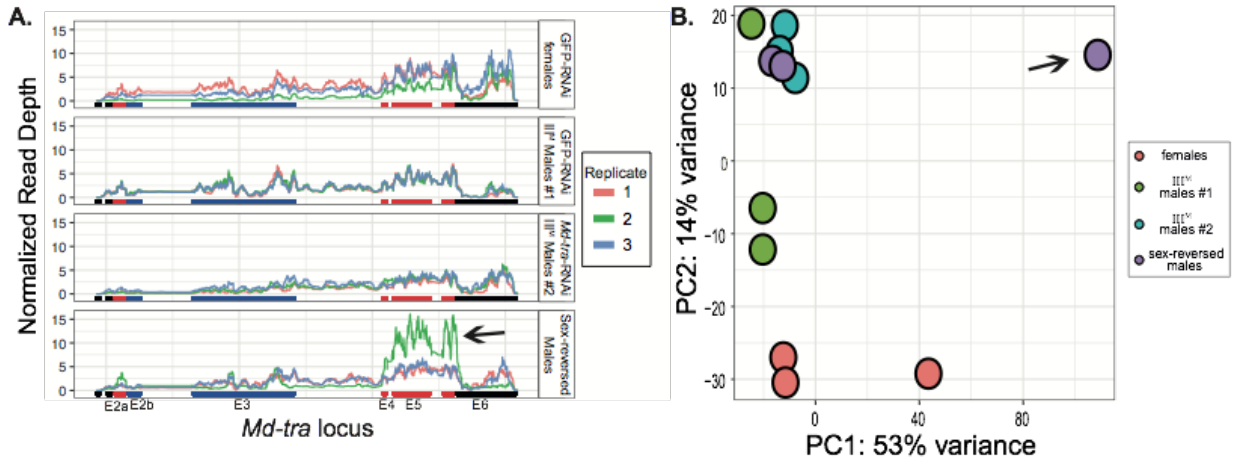
The expression of *Md-fru* was higher in head than in abdomen (**E-F**), consistent with its role as a behavioral regulator (Meier *et al.* 2013). Md-TRA regulates the splicing of *Md-fru* by promoting the production of splice variants with premature stop codons in females (Heinrichs *et al.* 1998; Meier *et al.* 2013). Sex-specific splicing of *Md-fru* occurs at the 5' end of the transcript (Meier *et al.* 2013), but the 5' end of *Md-fru* was not completely assembled and annotated in the reference genome. We therefore cannot test for differential splicing of *Md-fru* between males and females. However, we expect expression of *Md-fru* to be higher in males than females because the female splice variants will be removed by the NMD pathway. We indeed observe that *Md-fru* expression was much higher in the heads of GFP-RNAi treated genotypic males (III^M males #1; see Table 1) than GFP-RNAi treated normal females (**E-F**). However, in *Md-tra*-RNAi treated genotypic males (III^M males #2; see Table 1) and sex-reversed males, the expression of *Md-fru* is intermediate between females and GFP-RNAi treated genotypic males (**F**). A possible explanation is that RNAi knock down of *Md-tra* affects the expression or splicing of *Md-fru* in these flies (sex-reversed males and III^M males #2), but testing this hypothesis is beyond the scope of the work presented here.



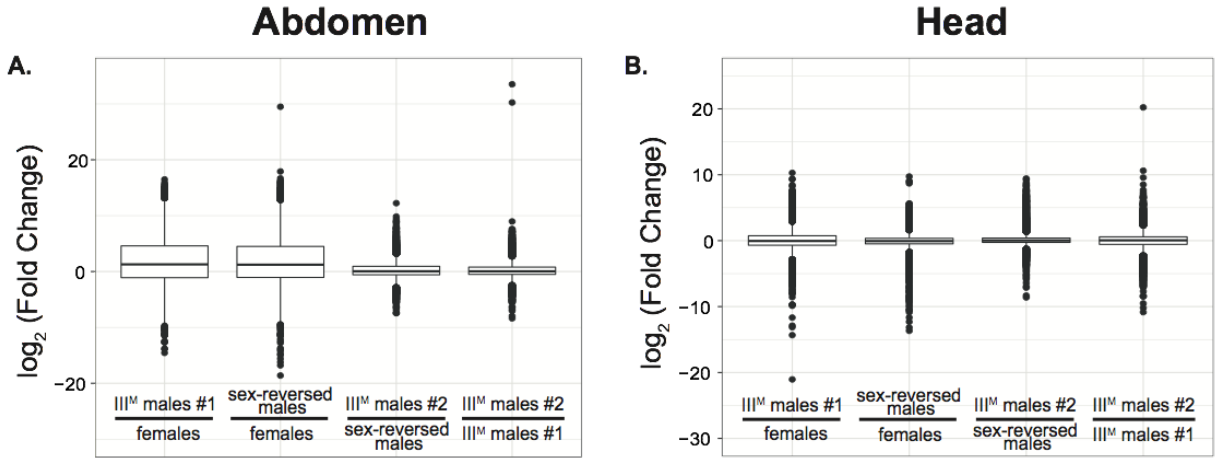
Supplementary Figure 8. Grade of membership model ($K = 3$) for gene expression patterns of four types of dsRNA injected flies in abdomens (**A**) and heads (**B**). Each row is one replicate of each genotype-by-treatment combination. Each color represents the proportion of each replicate assigned to each of the three clusters.



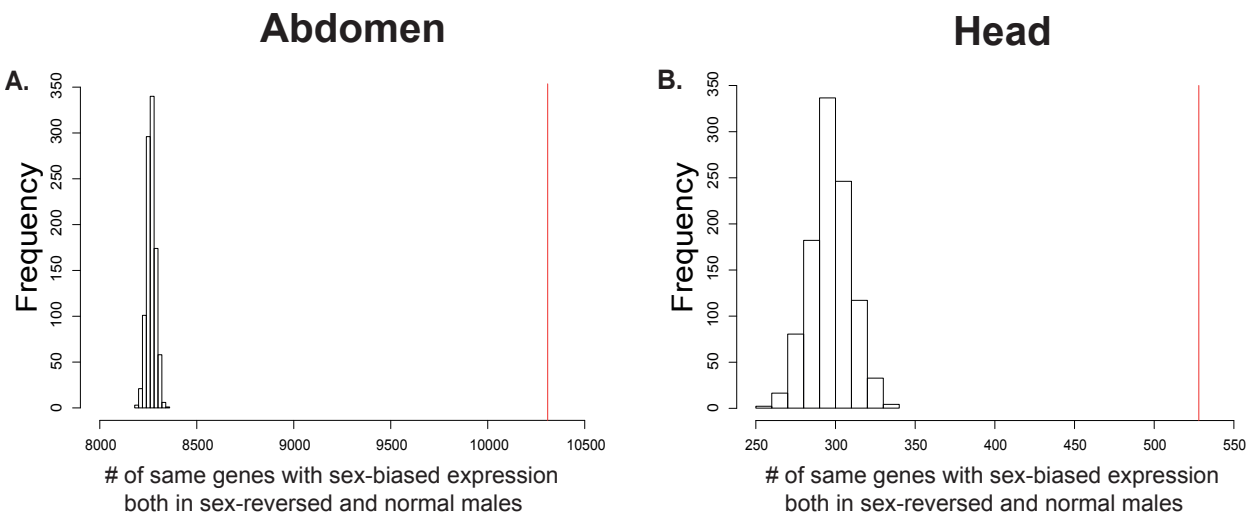
Supplementary Figure 9. Hierarchical clustering of RNAi-treated flies in abdomen (**A**) and head (**B**). GFP-female stands for GFP-RNAi normal females, GFP-male for GFP-RNAi normal males #1, Mdtra-N-male for *Md-tra*-RNAi normal males #2, and Mdtra-SR-male for *Md-tra*-RNAi sex-reversed males.



Supplementary Figure 10. *Md-tra* expression (**A**) and PCA of global expression (**B**) of GPF-RNAi and *Md-tra*-RNAi individuals in heads. Arrows indicate the sex-reversed male head sample that we excluded from our analysis because of its outlier expression profile. Females are GFP-RNAi Normal Females; III^M males #1 are GFP-RNAi Normal Males; III^M males #2 are *Md-tra*-RNAi Normal Males; sex-reversed males are *Md-tra*-RNAi Sex-Reversed Males. SR stands for sex-reversed.



Supplementary Figure 11. Boxplots show fold changes of gene expression among comparisons in abdomens (**A**) and heads (**B**). Outliers are included as points.



Supplementary Figure 12. Permutation tests for whether the same genes have sex-biased expression both in sex-reversed males and normal males (III^M males #1) in abdomens (**A**) and heads (**B**). Histograms represent null distribution and red lines indicate the observed number of genes with the same sex-biased expression both in sex-reversed and normal males.

strain	sex	tissue	Reads mapped	Total reads	% mapped reads
bwbCS	male	abdomen	28,797,051	53,216,164	54.11
bwbCS	male	abdomen	73,634,683	117,292,281	62.78
bwbCS	male	abdomen	34,387,641	46,794,091	73.49
CS	male	abdomen	23,436,748	43,892,080	53.40
CS	male	abdomen	47,225,783	73,027,117	64.67
CS	male	abdomen	29,656,467	39,569,021	74.95
CSrab	male	abdomen	26,931,208	44,702,325	60.25
CSrab	male	abdomen	10,639,480	17,154,576	62.02
CSrab	male	abdomen	39,105,284	51,590,908	75.80
IsoCS	male	abdomen	20,125,936	32,675,559	61.59
IsoCS	male	abdomen	5,422,571	8,475,350	63.98
IsoCS	male	abdomen	65,535,221	85,353,585	76.78
IsoCS	female	abdomen	49,248,555	61,591,761	79.96
CSrab	female	abdomen	24,268,638	33,301,977	72.87
bwbCS	male	head	41,466,785	55,597,781	74.58
bwbCS	male	head	36,041,171	50,840,587	70.89
bwbCS	male	head	62,840,805	80,615,028	77.95
CS	male	head	38,149,107	50,861,349	75.01
CS	male	head	19,919,990	27,227,824	73.16
CS	male	head	51,341,822	67,253,493	76.34
CSrab	male	head	56,191,834	75,651,390	74.28
CSrab	male	head	10,481,663	17,377,586	60.32
CSrab	male	head	55,120,600	73,965,574	74.52
IsoCS	male	head	17,332,681	27,276,701	63.54
IsoCS	male	head	28,841,223	39,570,265	72.89
IsoCS	male	head	62,213,672	82,095,892	75.78
IsoCS	female	head	49,089,361	63,708,772	77.05
CSrab	female	head	45,789,698	61,740,463	74.16
CS	female	head	45,947,520	57,865,112	79.40

Supplementary Table 1. Number and percent of RNA-seq reads mapping to the house fly reference genome for females and each male with a different naturally occurring proto-Y chromosome.

Sample Name	Reads mapped	Total reads	% mapped reads
Abdomen samples			
GFP-female-A2	91,396,275	106,578,289	85.76
GFP-female-A3	79,790,013	93,362,685	85.46
GFP-female-A4	67,507,547	78,662,688	85.82
GFP-male-A1	30,825,624	41,147,995	74.91
GFP-male-A2	43,102,152	57,473,417	74.99
GFP-male-A3	43,544,801	59,178,052	73.58
tra-N-A2	36,982,401	52,453,415	70.51
tra-N-A3	68,463,509	96,288,121	71.10
tra-N-A4	34,147,545	48,784,891	70.00
tra-SR-A1	45,637,174	60,334,091	75.64
tra-SR-A2	54,578,379	72,306,455	75.48
tra-SR-A3	35,999,042	47,579,279	75.66
Head samples			
GFP-female-H2	41,898,942	53,096,665	78.91
GFP-female-H3	54,190,305	69,827,731	77.61
GFP-female-H4	68,366,760	90,002,468	75.96
GFP-male-H1	86,270,347	106,498,553	81.01
GFP-male-H2	76,486,314	96,639,044	79.15
GFP-male-H3	105,491,479	132,492,819	79.62
tra-N-H2	42,279,891	55,339,089	76.40
tra-N-H3	22,602,895	29,743,740	75.99
tra-N-H4	31,937,609	41,479,997	77.00
tra-SR-H1	47,090,731	60,413,724	77.95
tra-SR-H2	82,077,805	101,558,122	80.82
tra-SR-H3	25,046,702	31,679,494	79.06

Supplementary Table 2. Number and percent of RNA-seq reads mapping to the house fly reference genome for each sample from the RNAi experiment.

Tissue	Comparison	#Diff	#Genes	Freq Diff
Abdomen	Y^M vs Y^M with new chr III	1159	11533	0.100
	Y^M vs III^M (CS)	511	10344	0.049
	Y^M vs III^M (CSrab)	479	9346	0.051
	III^M (CS) vs III^M (CSrab)	196	10460	0.19
Head	Y^M vs Y^M with new chr III	878	11909	0.074
	Y^M vs III^M (CS)	377	11845	0.032
	Y^M vs III^M (CSrab)	525	12390	0.042
	III^M (CS) vs III^M (CSrab)	739	12409	0.060

Supplementary Table 3. Differential expression between males with different genotypes. Counts of the number of genes that are expressed differentially (# Diff) and total genes expressed (#Genes) are shown, as well as the frequency of genes that are expressed differentially (Freq Diff). Y^M males are from the IsoCS strain; bwbCS Y^M males have a standard chromosome III from CS (bwbCS×CS males); III^M males are from either the CS or CSrab strain.

Tissue	Comparison	#Diff	#Genes	Freq Diff
Abdomen	III^M males #1 vs females	11030	14993	0.736
	sex-reversed males vs females	11005	14686	0.749
	III^M males #2 vs sex-reversed males	2867	13769	0.208
	III^M males #2 vs III^M males #1	2243	13162	0.170
Head	III^M males #1 vs females	5077	13558	0.374
	sex-reversed males vs females	735	12360	0.059
	III^M males #2 vs sex-reversed males	204	12959	0.016
	III^M males #2 vs III^M males #1	3260	13258	0.246

Supplementary Table 4. Differential expression between genotypic males and females with different RNAi treatments. Counts of the number of genes that are expressed differentially (#Diff) and total genes expressed (#Genes) are shown, as well as the frequency of genes that are expressed differentially (Freq Diff). Females are GFP-RNAi treated normal females; sex-reversed males are *Md-tra*-RNAi treated genotypic females; III^M males #1 are GFP-RNAi treated genotypic males; III^M males #2 are *Md-tra*-RNAi treated genotypic males.

Abdomen (# genes)		sex-reversed males vs females			
		male-biased	not female-biased	not male-biased	female-biased
genotypic male (III^M males #1) vs females	male-biased	6136	407	19	1
	not female-biased	483	1574	271	5
	not male-biased	26	274	841	182
	female-biased	5	18	277	4167
Head (# genes)		sex-reversed males vs females			
		male-biased	not female-biased	not male-biased	female-biased
genotypic male (III^M males #1) vs females	male-biased	254	1897	380	4
	not female-biased	50	2221	1177	34
	not male-biased	13	1091	2660	110
	female-biased	3	154	2045	267

Supplementary Table 5. Genes with sex-biased expression in sex-reversed or genotypic males. Counts are the number of genes that belong to each column and row combination. Columns compare sex-reversed males and normal females. Rows compare genotypic (normal) males and normal females. Genes with male-biased (female-biased) expression are expressed at significantly different levels between the sexes. Genes with not female-biased (not male-biased) have $\log_2$M/F not greater (less) than zero.

		Abdomen					
		normal-up-discordant (n-u-d)			sex-reversed-up-discordant (sr-u-d)		
Chromosomes (Muller elements)	# genes on chr	# genes	Odds ratio	95% CI	# genes	Odds ratio	95% CI
1(B)	2000	1	0.270	0.006 - 1.709	2	0.270	0.031 - 1.048
2(E)	2910	4	0.806	0.194 - 2.532	11	1.231	0.550 - 2.568
3(A)	**2094**	**9**	**4.129**	**1.482 - 11.322**	**13**	**2.386**	**1.119 - 4.853**
4(D)	2184	3	0.817	0.152 - 2.58	5	0.660	0.201 - 1.705
5(C)	2469	2	0.440	0.049 - 1.855	7	0.844	0.313 - 1.958
X(F)	45	0	0	0 - 57.539	0	0	0 - 27.459
Total	11702	19			38		

Supplementary Table 6. Chromosomal distribution of discordant sex-biased genes in abdomen. The chromosomal distribution of discordant genes was compared to all genes in the genome. Genes that were not assigned to a chromosome were excluded. A Fisher's exact test was performed to test for an excess of discordant sex-biased genes on each chromosome relative to genes on all other chromosomes.

		Head					
		normal-up-discordant (n-u-d)			sex-reversed-up-discordant (sr-u-d)		
Chromosomes (Muller elements)	# genes on chr	# genes	Odds ratio	95% CI	# genes	Odds ratio	95% CI
1(B)	1750	67	1.145	0.859 - 1.507	18	0.862	0.490 - 1.436
2(E)	2550	73	0.792	0.601 - 1.032	24	0.759	0.463 - 1.201
3(A)	**1842**	**108**	**2.028**	**1.592 - 2.569**	**44**	**2.665**	**1.787 - 3.933**
4(D)	1888	50	0.735	0.531 - 0.999	12	0.494	0.247 - 0.902
5(C)	2147	51	0.641	0.465 - 0.868	21	0.805	0.476 - 1.303
X(F)	34	1	0.858	0.021 - 5.144	0	0	0 - 9.947
Total	10211	350			119		

Supplementary Table 7. Chromosomal distribution of discordant sex-biased genes in head. The chromosomal distribution of discordant genes was compared to all genes in the genome. Genes that were not assigned to a chromosome were excluded. A Fisher's exact test was performed to test for an excess of discordant sex-biased genes on each chromosome relative to genes on the other chromosomes.

References cited

Dübendorfer A., and M. Hediger, 1998 The female-determining gene *F* of the housefly, *Musca domestica*, acts maternally to regulate its own zygotic activity. Genetics 150: 221–226.

Goldman T. D., and M. N. Arbeitman, 2007 Genomic and functional studies of Drosophila sex hierarchy regulated gene expression in adult head and nervous system tissues. PLoS Genet. 3: e216.

Hediger M., G. Burghardt, C. Siegenthaler, N. Buser, D. Hilfiker-Kleiner, *et al.*, 2004 Sex determination in *Drosophila melanogaster* and *Musca domestica* converges at the level of the terminal regulator *doublesex*. Dev. Genes Evol. 214: 29–42.

Hediger M., C. Henggeler, N. Meier, R. Perez, G. Saccone, *et al.*, 2010 Molecular characterization of the key switch *F* provides a basis for understanding the rapid divergence of the sex-determining pathway in the housefly. Genetics 184: 155–170.

Heinrichs V., L. C. Ryner, and B. S. Baker, 1998 Regulation of sex-specific selection of fruitless 5' splice sites by transformer and transformer-2. Mol. Cell. Biol. 18: 450–458.

Kervestin S., and A. Jacobson, 2012 NMD: a multifaceted response to premature translational termination. Nat. Rev. Mol. Cell Biol. 13: 700–712.

Meier N., S. C. Käppeli, M. Hediger Niessen, J.-C. Billeter, S. F. Goodwin, *et al.*, 2013 Genetic control of courtship behavior in the housefly: evidence for a conserved bifurcation of the sex-determining pathway. PLoS One 8: e62476.

Meisel R. P., J. G. Scott, and A. G. Clark, 2015 Transcriptome Differences between Alternative Sex Determining Genotypes in the House Fly, Musca domestica. Genome Biol. Evol. 7: 2051–2061.

Robinett C. C., A. G. Vaughan, J.-M. Knapp, and B. S. Baker, 2010 Sex and the single cell. II. There is a

time and place for sex. PLoS Biol. 8: e1000365.