**Temperature-dependent effects of house fly proto-Y chromosomes on gene expression act independently of the sex determination pathway**

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**Abstract**

Sex determination, the developmental process by which sexually dimorphic phenotypes are established, evolves fast. Species with polygenic sex determination, in which master regulatory genes are found on multiple different proto-sex chromosomes, are informative models to study the evolution of sex determination. House flies are such a model system, with male determining loci possible on all six chromosomes and a female-determiner on one of the chromosomes as well. The distributions of the two most common male-determining proto-Y chromosomes across natural populations suggests that temperature variation is an important selection pressure responsible for maintaining polygenic sex determination in this species. To test that hypothesis, we used RNA-seq to identify temperature-dependent effects of the proto-Y chromosomes on gene expression. We find no evidence for ecologically meaningful temperature-dependent expression of sex determining genes between male genotypes, but we identified hundreds of other genes whose expression depends on the interaction between proto-Y chromosome genotype and temperature. Notably, genes with genotype-by-temperature interactions on expression are not enriched on the proto-sex chromosomes. Moreover, there is no evidence that temperature-dependent expression is driven by chromosome-wide expression divergence between the proto-Y and proto-X alleles. Therefore, if temperature-dependent gene expression is responsible for differences in phenotypes and fitness of proto-Y genotypes across house fly populations, these effects are driven by a small number of temperature-dependent alleles on the proto-Y chromosomes.

## Introduction

Sex determination establishes sexually dimorphic developmental pathways, either based on genetic differences between males and females or environmental cues (Beukeboom & Perrin, 2014). In species with genotypic sex determination, a single master regulatory locus (e.g., *SRY* on the human Y chromosome) is often enough to initiate whether the embryo develops into a male or female (Goodfellow & Lovell-Badge, 1993; Sinclair et al., 1990). However, in polygenic sex determination systems, multiple master sex determining loci segregate independently, often on different chromosomes (Moore & Roberts, 2013). Polygenic sex determination is predicted to be an evolutionary intermediate between monogenic sex determination systems, and the factors responsible for maintaining polygenic sex determination as a stable polymorphism are poorly understood (Rice, 1986; G. Sander van Doorn, 2014). Understanding how polygenic sex determination is maintained would provide valuable insight into the factors that drive the evolution of sex determination.

House fly (*Musca domestica*) has a stable polygenic sex determination system with multiple male and female determining loci segregating in natural populations (Hamm, Meisel, & Scott, 2015). Male sex in house fly is initiated by the gene *Musca domestica male determiner, Mdmd* (Sharma et al., 2017). *Mdmd* is a recent duplication of the ubiquitous splicing factor *nucampholin* (*Md-ncm*). *Mdmd* promotes male development by causing the house fly ortholog of *transformer* (*Md-tra*) to be spliced into non-functional isoforms with premature stop codons (Monika Hediger et al., 2010). The lack of functional Md-Tra leads to male-specific splicing of *doublesex* (*Md-dsx*) and *fruitless* (*Md-fru*), the two known downstream targets of *Md-tra* (Monika Hediger et al., 2004; Meier et al., 2013). In the absence of *Mdmd*, *Md-tra* is spliced into a functional transcript that is translated into a protein that promotes female specific splicing of *Md-dsx* and inhibits splicing of the male isoform of *Md-fru*.

*Mdmd* can be found on multiple different chromosomes in house fly (Sharma et al., 2017), and it is most commonly found on the third ($III^M$) and Y ($Y^M$) chromosomes (Hamm et al., 2015). These *Mdmd*-bearing chromosomes are young proto-Y chromosomes that are minimally differentiated from their homologous X chromosomes (Meisel, Gonzales, & Luu, 2017). The proto-Y chromosomes are clinally distributed—with $III^M$ most common at southern latitudes and $Y^M$ most common at northern latitudes—across multiple continents (Denholm, Franco, Rubini, & Vecchi, 1986; Hamm, Shono, & Scott, 2005; Hiroyoshi, 1964; Mcdonald et al., 1975). The frequencies of $III^M$ and $Y^M$ in natural populations have remained stable for decades, suggesting

that natural selection maintains the polymorphism (Kozielska, Feldmeyer, Pen, Weissing, & Beukeboom, 2008; Meisel et al., 2016).

The natural distribution of $III^M$ and $Y^M$ hints at a possible genotype-by-temperature (GxT) interaction that could explain the stable maintenance of $Y^M$-$III^M$ clines. Indeed, seasonality in temperature is the best predictor of the distribution of the proto-Y chromosomes (Feldmeyer et al., 2008). Polygenic sex determination in house fly could therefore be maintained in a similar way to how temperature variation maintains opposing clines of heat and cold tolerance in *Drosophila melanogaster* between tropical and temperate regions (Hoffmann, Anderson, & Hallas, 2002). In addition, in some fish and reptile species, temperature can drive sex determination and override the outcomes of genotypic sex determining systems (Holleley et al., 2015; Quinn et al., 2007; Radder, Quinn, Georges, Sarre, & Shine, 2008; Shine, Elphick, & Donnellan, 2002), providing an intriguing functional link between temperature variation and sex determination.

If polygenic sex determination in house fly is maintained by temperature-dependent selection pressures, we expect the phenotypes and fitness of $III^M$ and $Y^M$ males to vary across temperatures. These temperature-dependent effects could be driven by differential gene expression in $III^M$ and $Y^M$ males at across temperatures. Here, we test that hypothesis by evaluating how GxT interactions affect gene expression in male house flies carrying either the $III^M$ or $Y^M$ proto-Y chromosome. We used RNA-seq to study gene expression in two nearly isogenic lines of house flies, differing only by their proto-Y chromosome, reared at two developmental temperatures. We also used quantitative reverse transcription PCR (qRT-PCR) to investigate the temperature-dependent expression of *Mdmd*.

## Materials & Methods

### *qRT-PCR samples and analysis*

We used qRT-PCR to measure the expression of *Mdmd* and its paralog *Md-ncm* in two $Y^M$ strains and two $III^M$ strains, which were grouped into two pairs (one $Y^M$ strain and one $III^M$ strain per pair). In the first pair, we used the $Y^M$ strain IsoCS and the $III^M$ strain CSkab (both from North America). IsoCS and CSkab share a common genetic background of the Cornell susceptible (CS) strain (J. G. Scott, Sridhar, & Liu, 1996). IsoCS was previously created by crossing a $Y^M$ chromosome from Maine onto the CS background (Hamm, Gao, Lin, & Scott, 2009). We created CSkab by backcrossing the $III^M$ chromosome from the KS8S3 strain collected in Florida (Kaufman, Nunez, Geden, & Scharf, 2010) onto the CS background, using an

approach described previously (Son et al., 2019). In the second pair, we used two European strains: the $Y^M$ strain GK-1 from Gerkesklooster (Netherlands) and the $III^M$ strain SPA3 from near Girona (Spain).

We raised all strains at 18°C and 27°C for two generations with 12:12-h light:dark photoperiods. Adult males and females for each GxT combination were housed in cages with *ad libitum* containers of 1:1 combinations of sugar and non-fat dry milk and *ad libitum* containers of water. Females were provided with a standard medium of wheat bran, calf manna, wood chips, yeast, and water in which to lay eggs for 12-24 hrs (Hamm et al., 2009). The resulting larvae were maintained in the same media within 32 oz containers. Adult females were unable to lay eggs at 18°C, so the adults from the 18°C colonies were transferred to 22°C for egg laying for 1-2 days. The eggs collected at 22°C were then moved back to 18°C for larval development, pupation, and emergence as adults. We maintained the colonies at these temperatures for two generations. Collecting flies after two generations ensured at least one full egg-to-adult generation at the appropriate temperature.

For qRT-PCR experiments involving the North American IsoCS and CSkab strains, abdomen samples were dissected from 5 day old adult males after being anesthetized with $CO_2$. For qRT-PCR assessments on the European GK and SPA3 strains, full body samples were collected from 5 day old adult males after being anesthetized with $CO_2$. Tissue samples from 5-7 males were pooled in each of three biological replicates for each genotype ($Y^M$ and $III^M$) by temperature (18°C and 27°C) combination. The collected tissues were homogenized in TRIzol reagent (Life Technologies) using a motorized grinder in a 1.5mL microcentrifuge tube. For the North American strains, the Direct-zol RNA MiniPrep kit (Zymo Research) was used to extract RNA from the homogenized samples. The isolated RNA was reverse transcribed into cDNA with MLV RT (Promega), following the manufacturer's protocol. For the European strains, the RNA phase following centrifugation with TRIzol reagent was separated using chloroform and precipitated by using isopropanol and ethanol. The isolated RNA was reverse transcribed into cDNA using RevertAid H minus 1st strand kit (Fermentas #K1632) according to the manufacturer's protocol.

We conducted qRT-PCR of cDNA from the male flies. We used qRT-PCR primers (Supplementary table 1) to uniquely amplify *Mdmd* and *Md-ncm* without amplifying the other paralog (Sharma et al., 2017). Primers were additionally used to amplify cDNA from a transcript (LOC101888902) that is not differentially expressed between $Y^M$ and $III^M$ males as an internal control for cDNA content in each biological replicate (Meisel, Scott, & Clark, 2015). The IsoCS

and CSkab samples were assayed on a StepOnePlus machine using PowerUp SYBR Green Master Mix (Applied Biosystems). The GK and SPA3 samples were assayed on a Applied Biosystems qPCR cycler 7300 machine using Quanta perfecta SYBR Green Fastmix (Quanta bio). We measured the abundance of PCR products from each primer pair in three technical replicates of three biological replicates for each GxT combination. With the same primer pairs, we also measured the expression of serial dilutions (1/1, 1/5, 1/25, 1/125, and 1/625) of cDNA from independent biological collections of house flies. Samples were interspersed across 96-well microtiter plates to minimize batch effects.

We constructed standard curves for each primer pair by calculating the linear relationship between CT values and $\log_{10}$(concentration in the serial dilution) using the lm() function in the R statistical programming package (R Core Team, 2019). We then used the equations of the standard curves to calculate the concentration of transcripts (i.e., cDNA) from *Mdmd* and *Md-ncm* in each technical replicate. We next determined a normalized expression level of each technical replicate by dividing the concentration of the technical replicate by the mean concentration of the control transcript (LOC101888902) across the three technical replicates from the same biological replicate.

We used an analysis of variance (ANOVA) approach to test for the effect of genotype ($Y^M$ vs $III^M$), developmental temperature (18°C vs 27°C), and the interaction of genotype and temperature on the expression of each transcript. To those ends, we used the lmer() function in the lme4 package (Bates, Mächler, Bolker, & Walker, 2015) in R to model the effect of genotype ($G$), temperature ($T$), and the interaction term as fixed effect factors, as well as biological replicate ($r$) as a random effect, on expression level ($E$):

$$E \sim G + T + G \times T + r.$$

We then compared the fit of that full model to a model without the interaction term ($E \sim G + T + r$) using the anova() function in R. If the full model fits significantly better, that is evidence that there is a significant GxT interaction on the expression of the transcript.

***RNA-seq samples***

We used RNA-seq to measure gene expression in a $Y^M$ strain known as IsoCS and a $III^M$ strain known as CSrab. IsoCS (described above) and CSrab have different proto-Y chromosomes on the shared CS genetic background (J. G. Scott et al., 1996). We created CSrab by backcrossing the $III^M$ chromosome of a spinosad-resistant strain, rspin (Shono &

Scott, 2003), onto the CS background, using the same approach as we used to create CSkab, described elsewhere (Son et al., 2019).

Colonies of both strains were reared at 18°C and 29°C for two generations with at least one full egg-to-adult generation, as described above. We controlled for the adult density using 35 adult males and 35 adult females for each GxT combination. We also controlled for larval density with 100 larvae per 32 oz container. Third generation males obtained from second generation females were collected and reared separately from the females at their respective developmental temperatures for 1-8 days before RNA extraction.

For the RNA-seq experiments, head and testis samples from 1-8 day old males were dissected in 1% PBS solution after being anesthetized with $CO_2$. We dissected testes from 15-20 house flies per each of three replicates of each GxT combination. Similarly, 5 heads were dissected for each of three biological replicates for each GxT combination. The collected tissues were homogenized in TRIzol reagent (Life Technologies) using a motorized grinder in a 1.5 mL microcentrifuge tube. The Direct-zol RNA MiniPrep kit (Zymo Research) was used to extract RNA from the homogenized samples. RNA-seq library preparation was carried out using the TruSeq Stranded mRNA Kit (Illumina). Qualities of these libraries were assessed using a 2100 Bioanalyzer (Agilent Technologies, Inc.). Libraries were then sequenced with 75 bp single-end reads on high output runs of an Illumina NextSeq 500 at the University of Houston Seq-N-Edit Core. All testis samples (i.e., all replicates of each GxT combination) were sequenced together in a single run, and all head samples were sequenced together on a separate run. All RNA-seq data are available in the NCBI Gene Expression Omnibus under accession GSE136188 (BioProject PRJNA561541, SRA accession SRP219410).

***RNA-seq data analysis***

RNA-seq reads were aligned to the house fly reference genome Musca_domestica-2.0.2 (Jeffrey G. Scott et al., 2014) using HISAT2 (Kim, Langmead, & Salzberg, 2015). We next used SAMtools (Li et al., 2009) to sort the aligned reads. The sorted reads were assigned to annotated genes (*M. domestica* Annotation Release 102) using htseq-count (Anders, Pyl, & Huber, 2015). We analyzed the exon-level expression of the sex determining genes *Md-tra* (LOC101888218) and *Md-dsx* (LOC101895413) for each GxT combination. To do so, we calculated normalized allele depth ($D_{ijk}$) at each site $i$ within each gene in library $j$ for GxT combination $k$ by dividing the number of reads mapped to a site ($r_{ijk}$) into the total number of reads mapped in that library ($R_{jk}$), and we multiplied that value by one million:

$$D_{ijk} = \left(\frac{r_{ijk}}{R_{jk}}\right) 10^6 .$$

For each site within each gene, we then calculated the average $D_{ijk}$ across all three libraries for each GxT combination.

We also used the DESeq2 package in R (Love, Huber, & Anders, 2014) to analyze differential expression of all annotated genes between all GxT combinations. To do so, we used a linear model that included genotype ($G$, which can be $Y^M$ or $III^M$), developmental temperature ($T$), and their interaction term to predict gene expression levels ($E$):

$$E \sim G + T + G \times T .$$

Genes for which the interaction term has a false discovery rate (FDR) corrected *P*-value (Benjamini & Hochberg, 1995) of less than 0.05 were considered to be differentially expressed as a result of the GxT interaction. The same FDR corrected cutoff was used to test for genes that are differentially expressed according to genotype or temperature, by testing for the effect of $G$ or $T$ using results analyzed with the full model. For principal component analysis (PCA) and hierarchical clustering, we analyzed regularized log transformed count data generated by the rlog() function in DESeq2. Non-metric multidimensional scaling (NMDS) of normalized count data obtained from DESeq2 was carried out using the vegan package in R (Oksanen et al., 2019).

We performed a gene ontology (GO) analysis to test for enrichment of functional classes amongst differentially expressed genes. To assign GO terms to house fly genes, we first used BLASTX to search house fly transcripts against a database of all *D. melanogaster* proteins (Gish & States, 1993). We took this approach because GO assignments are missing for most house fly genes. The top hit for each house fly gene obtained from BLASTX were used to assign flybase IDs to homologous house fly transcripts. These *D. melanogaster* homologs were then used in DAVID 6.8 (Huang, Sherman, & Lempicki, 2009a, 2009b) to identify GO terms that are significantly enriched amongst differentially expressed genes (FDR corrected $P < 0.05$).

***Allele-specific expression analysis***

We tested for differential expression of third chromosome genes between the allele on the $III^M$ chromosome and the allele on the standard third chromosome in $III^M$ males. To do so, we first followed the Genome Analysis Toolkit (GATK) best practices workflow for single nucleotide polymorphism (SNP) and insertion/deletion (indel) calling to identify sequence variants in our RNA-seq data (McKenna et al., 2010). We first used STAR (Dobin et al., 2013) to

align reads from the 12 testis libraries and 12 head libraries to the house fly reference genome (Musca_domestica-2.0.2). We then used the splice junction information from the first alignment to create a new index that was used to perform a second alignment. Using *de novo* transcripts identified with STAR serves to remove any read-mapping biases associated with the incomplete transcript annotation. After adding read group information to the SAM file thus generated, we marked duplicates. We next used SplitNCigarReads to reassign mapping qualities to 60 with the ReassignOneMappingQuality read filter for alignments with a mapping quality of 255. We used RealignerTargetCreator to identify and IndelRealigner to realign the indels. We used BaseRecalibrator and variant calls from a previous RNA-seq analysis (R. P. Meisel et al., 2017) to recalibrate the realigned reads. The realigned reads were then used for variant calling with HaplotypeCaller with emission and calling threshold of 20. We filtered the variants obtained using VariantFiltration with a cluster window size of 35 bp, cluster size of 3 SNPs, FS > 30, and QD < 2. We then used all the generated gvcf files to carry out joint genotyping using GenotypeGVCFs. We performed separate joint genotyping for testis and head libraries. The variants from Joint Genotyping were then filtered using VariantFiltration with FS > 30 and QD < 2. We used the vcfR package in R (Knaus & Grünwald, 2017) to extract information from vcf files obtained from joint genotyping. For downstream analysis, we only kept SNPs (i.e., variants where the reference and alternate allele are 1 bp), and excluded small indels.

To test for allele-specific expression, we first assigned sequence variants to the $III^M$ and standard third (III) chromosomes. This was only done for sites that were heterozygous in $III^M$ males and homozygous in $Y^M$ males (all other variable sites on the third chromosome were discarded) because these are the only alleles we can assign to either the $III^M$ or III chromosome. This is because $Y^M$ males are homozygous for the III chromosome (X/$Y^M$; III/III), and $III^M$ males are heterozygous (X/X; $III^M$/III). For every variable site, we assigned the allele shared by both $III^M$ and $Y^M$ males to the III chromosome, and the allele unique to $III^M$ males to the $III^M$ chromosome. We calculated the sum of read depth for each allele across all three sequencing libraries (i.e., replicates) of each GxT combination. For each gene, we calculated the average normalized read depth across all variable sites within the gene separately for the $III^M$ and III alleles at each temperature. To compare the expression of the $III^M$ and III alleles, we calculated the difference in sequencing coverage between $III^M$ and III alleles at each site for each temperature separately. We calculated the average difference in expression of $III^M$ and III alleles in each gene at each temperature $k$, $d_k$, as follows:

$$d_k = \frac{1}{n}\sum_{i=1}^{n}\left(\frac{r_{i1k}}{R_k} - \frac{r_{i2k}}{R_k}\right)10^6,$$

where $r_{i1k}$ is the expression of the III$^M$ allele at site $i$ (out of $n$ total polymorphic sites) and temperature $k$ (either 18°C or 29°C), $r_{i2k}$ is the expression of the III allele at site $i$ and temperature $k$, and $R_k$ is the total number of mapped reads in III$^M$ males at temperature $k$. We then calculated standard error of $d_k$ across all sites for each gene at each temperature.

## Results

### *GxT interactions and the expression of genes in the house fly sex determination pathway*

We first tested the hypothesis that the fidelity of the house fly sex determination pathway depends on GxT interactions. Sex determination pathways can be temperature sensitive, which can allow for transitions between genotypic and temperature-dependent sex determination (Holleley et al., 2015; Quinn et al., 2007; Radder et al., 2008; Shine et al., 2002). It is therefore possible that temperature-dependent selection acts on allelic variation within the sex-determination pathway itself to maintain polygenic sex determination in house fly. Y$^M$ is found at northern (colder) latitudes, and III$^M$ is found at southern (warmer) latitudes (Feldmeyer et al., 2008; Hamm et al., 2005; Kozielska et al., 2008) in the Northern hemisphere. Sex determination in flies operates via sex-specific alternative splicing of genes in the sex determining pathway (Bopp, Saccone, & Beye, 2014; Salz, 2011), and the male-determiner *Mdmd* is a negative regulator of *Md-tra* splicing (Monika Hediger et al., 2010; Sharma et al., 2017). We thus hypothesize that *Mdmd* has more activity (possibly because of higher expression) or *Md-tra* is more accurately spliced in Y$^M$ males at low temperature and III$^M$ males at high temperature.

We used qRT-PCR to examine the expression of the male-determining gene, *Mdmd*, in two III$^M$ strains and two Y$^M$ strains raised at 18°C and 27°C (Supplementary Figure 1). One Y$^M$ strain and one III$^M$ strain were isolated from North America, and the other Y$^M$ strain and III$^M$ strain were isolated from Europe. There is a significant GxT interaction affecting the expression of *Mdmd* in the European Y$^M$ and III$^M$ strains (Supplementary Figure 1C). However, the direction of the effect is opposite of our hypothesized interaction—*Mdmd* is expressed higher in III$^M$ males at colder temperatures. A similar trend is observed in the North American strains, although the interaction term is not significant. We also did not find a significant GxT interaction affecting expression of *Md-ncm* (the ancestral paralog of *Mdmd*) , which is not part of the sex

determination pathway (Supplementary Figure 1). Therefore, there is no evidence that *Mdmd* expression is increased at the favored temperatures for $Y^M$ and $III^M$ males.

We next tested the hypothesis that GxT interactions affect the expression or splicing of *Md-tra (Monika Hediger et al., 2010; Sharma et al., 2017)*. Using RNA-seq data that we collected from $Y^M$ and $III^M$ males raised at 18°C and 29°C, we did not find significant differential expression of *Md-tra* because of GxT interactions in either head (Supplementary Table 2) or testis (Supplementary Table 3). If a GxT interaction affecting the splicing of *Md-tra* were responsible for the latitudinal distribution of $Y^M$ and $III^M$, we would expect more female-determining isoforms produced (i.e., misexpressed) in $Y^M$ males raised at a warm temperature, or higher expression of female-determining isoforms in $III^M$ males raised at a colder temperature. To test if a GxT interaction affects the usage of any of the *Md-tra* exons, we determined the normalized read depth across *Md-tra* exons in all GxT combinations of RNA-seq data from the heads and testes of house flies. *Md-tra* consists of exons with premature stop codons found in the non-functional male isoforms, and other exons found in both the female-determining isoform and non-functional male-isoforms (Monika Hediger et al., 2010). We refer to exons found in both the female-determining and male isoforms as “shared” exons (Supplementary Figure 2). If a GxT interaction affecting the splicing of *Md-tra* were responsible for the latitudinal distribution of $Y^M$ and $III^M$, we would expect higher usage of shared exons in $Y^M$ males raised at 29°C and $III^M$ males raised at 18°C, suggesting increased female-specific splicing of *Md-tra* at the discordant temperatures. We found significant effects of GxT interactions on the usage of most *Md-tra* exons in both head and testis, but these GxT interactions are not in the directions consistent with missplicing at discordant temperatures (Supplementary Figure 2). An analysis of *Md-tra* splicing with qPCR was not possible because we could not design primers that specifically amplified isoforms for quantitative assessment.

We further tested if GxT interactions affect the expression and splicing of two direct downstream targets of *Md-tra* in the sex determination pathway, *Md-dsx* and *Md-fru*. Our RNA-seq data show that there is no effect of GxT interactions in the expression of *Md-dsx* and *Md-fru* in head (Supplementary table 2) or testis (Supplementary table 3). We also found no evidence of GxT interactions affecting the expression of individual *Md-dsx* exons (Supplementary Figure 3). We did not test for GxT effects on the expression of *Md-fru* exons because exons that differentiate the male and female isoforms have not been annotated in the reference genome (Meier et al., 2013). We therefore did not find evidence that GxT interactions directly affect the expression of genes in the house fly sex determination pathway in a way that

is consistent with selection on the sex determination pathway being responsible for maintaining the $Y^M$-$III^M$ clines.

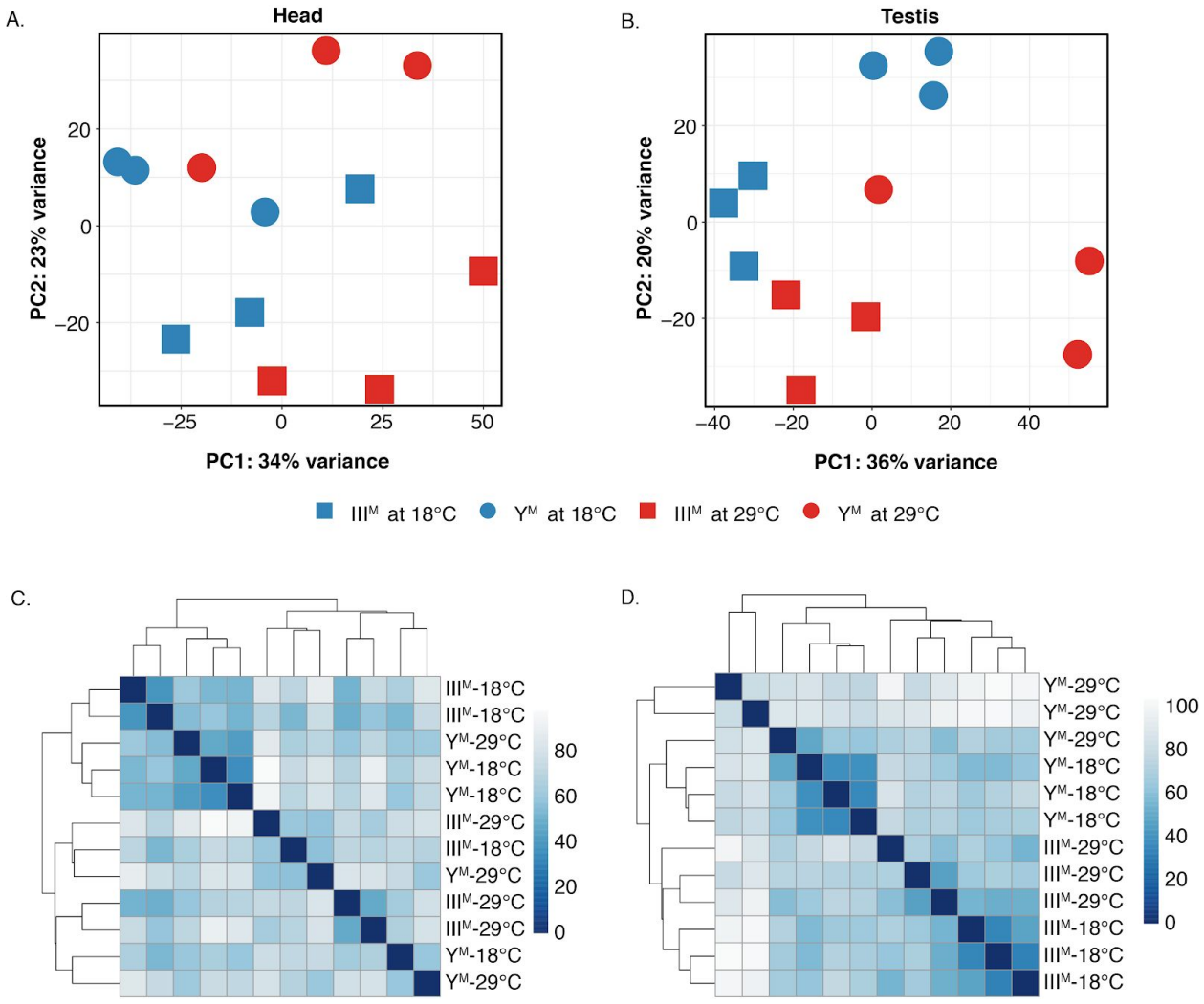


**Figure 1. Effect of genotype and temperature in genome-wide gene expression**. Principal component (PC) analysis showing overall gene expression pattern in head (A) and testis (B) samples. Each data point represents a biological replicate. Heatmaps and dendrograms showing hierarchical clustering in head (C) and testis (D) samples.

### *Genotype and temperature affect gene expression*

To test for GxT effects on the expression of genes not in the sex determination pathway, we analyzed RNA-seq data from heads and testes of $Y^M$ and $III^M$ house flies raised at 18°C and 29°C. We first used a PCA to assess the similarities of the overall gene expression profiles of each of three replicates of each GxT combination in head and testis separately (using all 16,540

genes in the reference annotation). The first principal component (PC1) of head gene expression explains 34% of the variance in expression, and the second (PC2) explains 23% of the variation (Figure 1A). However, there is no clear grouping by genotype or temperature. In testis, PC1 explains 36% of the variance in expression, and it separates $III^M$ males at 18°C from $Y^M$ males at 29°C (Figure 1B). $III^M$ is found at southern, warmer temperatures, whereas $Y^M$ is found at northern, colder temperatures. PC1 for testis expression therefore differentiates the two genotypes at the temperatures that are opposite from their geographic distribution. PC2 explains 20% of the variation in testis expression and separates $III^M$ at 29°C from $Y^M$ at 18°C (Figure 1B). PC2, therefore, differentiates the two genotypes at the temperatures that are consistent with their geographic distribution. We also carried out PCA by considering only the 500 most variable genes in head and testis and observed the same patterns (Supplementary Figure 4). We additionally carried out PCA for genes on each chromosome, and the results for each chromosome were consistent with those across all chromosomes (Supplementary Figures 5 and 6). One biological replicate for CSrab head and testis at both temperatures came from older males (4-8 days old, as opposed to the other samples which were 1-3 days old), but we did not see a meaningful effect of age on gene expression in our PCA (Supplementary Figure 7).

To confirm the patterns we observed in the PCA, we also carried out NMDS and hierarchical clustering of the RNA-seq data. The groupings in NMDS are consistent with the PCA results (Supplementary Figure 8). In the hierarchical clustering, we did not observe clustering by genotype or temperature in the head samples, consistent with the PCA and NMDS (Figure 1C). For testis gene expression, we found some evidence for clustering first by genotype and then by temperature in the hierarchical clustering (Figure 1D), similar to the PCA. However, the concordance between clusters and GxT combinations is not perfect.

To further test for genotype- and temperature-dependent gene expression, we next identified differentially expressed genes i) between genotypes at one developmental temperature (either at 18°C or 29°C) and ii) within a genotype across the two developmental temperatures. Comparing between genotypes, we found 900 genes that are differentially expressed between $Y^M$ and $III^M$ heads at 18°C, and there were 1378 genes differentially expressed between $Y^M$ and $III^M$ heads at 29°C (Supplementary Table 2). The number of differentially expressed genes is higher in testis than head: 2413 genes at 18°C and 2199 genes at 29°C are significantly differentially expressed between $Y^M$ and $III^M$ testes (Supplementary Table 3). This is consistent with previous work that identified more genes differentially

expressed between $Y^M$ and $III^M$ males in testis than head (Meisel et al., 2015). In both head and testis, there is an excess of genes on the third chromosome that are significantly differentially expressed between genotypes at both 18°C and 29°C (Figure 2A). This is consistent with different third chromosome genotypes between strains, and suggestive of a *cis*-effect on gene expression levels (Meisel et al., 2015; Son et al., 2019).

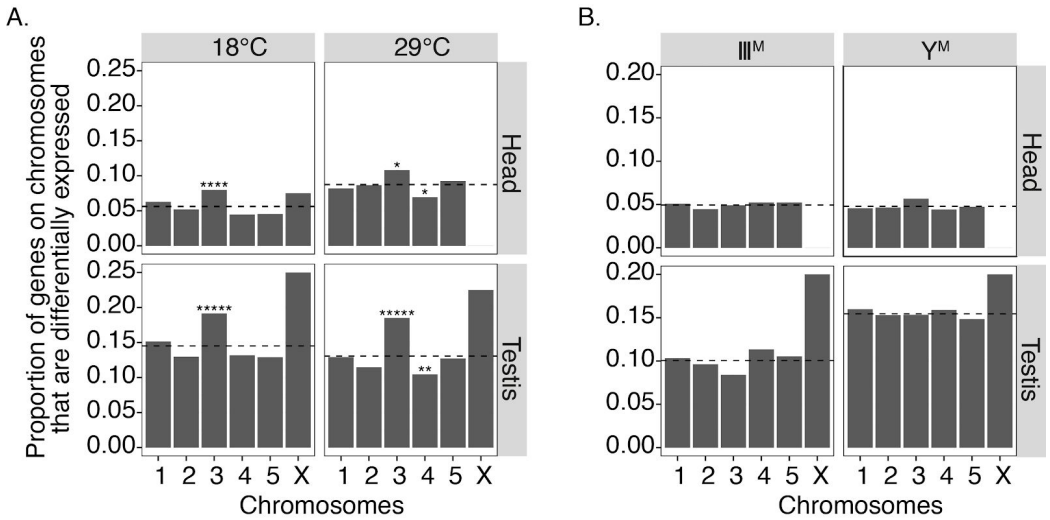


**Figure 2. Genes that are differentially expressed between genotypes are significantly enriched on the third chromosome.** A) The proportion genes that are differentially expressed between $Y^M$ and $III^M$ males is plotted for heads of flies raised at 18°C and heads at 29°C (top row), and testes of flies raised at 18°C at 29°C (bottom row). B) The proportion genes that are differentially expressed between temperatures are made for heads (top row) and testes (bottom row) for $III^M$ and $Y^M$ flies. Each bar represents the proportion of differentially expressed genes on a chromosome, and dashed lines show the genome-wide average. Asterisks indicate *P* values obtained from Fisher's exact test comparing the number of differentially expressed genes on a chromosome, the number of non-differentially expressed genes on a chromosome, and the number of differentially and non-differentially expressed genes across all other chromosomes, after Bonferroni correction (**P* < 0.05, ***P* < 0.005, ****P* < 0.0005, *****P* < 0.00005, ******P* < 0.000005).

When comparing between temperatures within each genotype, we found 739 genes significantly differentially expressed between heads of $Y^M$ flies raised at the two different temperatures (Supplementary Table 2). Similarly, 744 genes are differentially expressed between the heads of $III^M$ flies raised at different temperatures (Supplementary Table 2). In testis, there are 2402 genes in $Y^M$ flies and 1649 genes in $III^M$ flies that are differentially

expressed between 18°C and 29°C (Supplementary Table 3). There is no significant chromosomal enrichment of genes that are differentially expressed between temperatures in either head or testis (Figure 2B), consistent with these comparisons being between flies with the same genotype.

***GxT interactions affect the expression of a small subset of genes***

We next identified individual genes that are differentially expressed between $Y^M$ and $III^M$ males depending on temperature by testing for significant interactions between genotype and temperature on gene expression levels. We found 50 genes in head and 247 genes in testis whose expression significantly differs in response to GxT interactions (Supplementary Tables 2 and 3). We did not find an enrichment of genes with significant GxT interactions on any chromosome, in either head or testis (Supplementary Figure 9). Notably, there are 10 genes that are affected by GxT interactions in both head and testis. We would expect fewer than 1 gene to be affected by GxT interactions in both head and testis if the GxT effects are independent across tissues. The ten genes we observed are significantly more than this expectation ($z = 10.708$, $P < 0.00001$), suggesting GxT effects on expression are not independent across tissues. A similar non-independence of expression differences across tissues was previously observed between $Y^M$ and $III^M$ males (Meisel et al., 2015).

We further characterized the functional annotations of genes that are differentially expressed as a result of GxT interactions. Genes with significant GxT interactions in head are enriched for two GO terms: disulfide bond and signal. In head, we found a gene related to stress response (*apoliprotein-D*), a chemosensory gene (*odorant-binding protein 56h*), and immune genes (*defensin-2* and *lysozyme 1*) were differentially expressed because of GxT interactions. Genes with significant GxT interactions in testis are enriched for the GO term glycosylation site: N-linked. In testis, genes with significant GxT effects on expression include those coding for proteins related to reproductive functions (*protamine*, *asunder*, *sarah*, and *farnesyl pyrophosphate synthase*). Metabolic genes (e.g., *hexokinase*, *fructose-1,6-bisphosphatase*, and *indole-3-acetaldehyde oxidase*), immune related genes (*gram-negative bacteria-binding protein 3* and *prophenoloxidase 2*), adult lifespan related genes (*pointed* and *cystathionine β-synthase*) are also differentially expressed in testis because of GxT effects.

***GxT interactions on gene expression are not driven by cis-regulatory divergence***

We next tested if chromosome-wide *cis*-regulatory divergence between the $III^M$ and standard third chromosome is responsible for temperature-dependent expression differences

between $III^M$ and $Y^M$ males. $III^M$ males are heterozygous ($III^M$/III) whereas $Y^M$ males are homozygous (III/III) for a standard third chromosome. If *cis*-regulatory alleles on the third chromosome are responsible for differential expression of third chromosome genes between $III^M$ and $Y^M$ males, the $III^M$ and III alleles should be differentially expressed in $III^M$ males. For example, if a gene is more highly expressed in $III^M$ males than $Y^M$ males, the $III^M$ allele of the gene should be more highly expressed than the III allele in $III^M$ males. The opposite would be true if $Y^M$ males have higher expression than $III^M$ males. We used this logic to test if GxT interactions on gene expression are the result of *cis*-regulatory divergence of third chromosome genes between the $III^M$ and III chromosomes. To do so, we asked if genes on the third chromosome that are significantly differentially expressed in head or testis because of GxT interactions have concordant differences in expression between the $III^M$ and III allele in $III^M$ males.

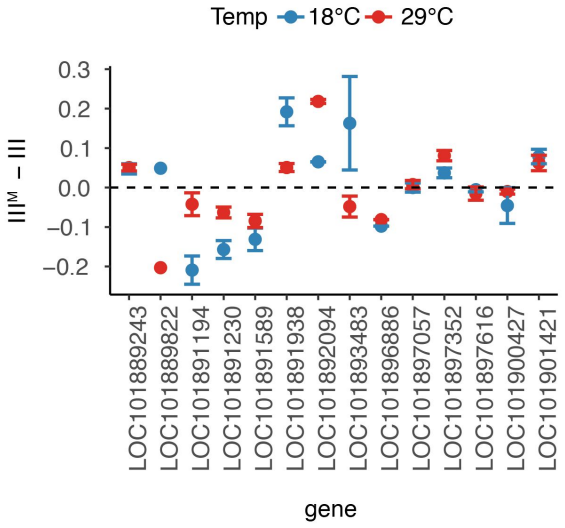


**Figure 3: GxT interactions on allele-specific expression.** Difference in sequencing coverage in $III^M$ males between $III^M$ and III alleles at 18°C and 29°C.

To test for differences in allelic expression, we first identified 14 genes on the third chromosome with a significant GxT interaction affecting testis expression, at least one heterozygous SNP in $III^M$ males, and homozygous at those SNP sites in $Y^M$ males (Figure 3). We required the variants to be heterozygous in $III^M$ males and homozygous in $Y^M$ males because we are interested in expression differences between the $III^M$ and III allele in $III^M$ males. We assumed that the allele in common between $III^M$ and $Y^M$ males is found on the standard third

chromosome, and the allele unique to $III^M$ males is on the $III^M$ chromosome. This assumption is reasonable because the $Y^M$ and $III^M$ flies share the same genetic background, and therefore should have the same standard third chromosome. We quantified the expression of the two alleles ($III^M$ and III) based on allele-specific RNA-seq read coverage. We asked if the difference in expression of $III^M$ alleles in each gene is consistent with the difference in overall expression of these genes between 18°C and 29°C within $III^M$ males. For example, if $III^M$ males have higher expression at 29°C, we expect the difference between the $III^M$ and III alleles to be greater at 29°C than 18°C.

**Table 1:** Temperature-dependent allele-specific expression across the genome.

| | 3rd Chromosome | Rest of genome |
|---|---|---|
| Genes with significant temperature effect on $III^M$ and $III^M$-III in right direction | 4 | 8 |
| Genes with significant temperature effect on $III^M$ and incorrect direction of $III^M$-III | 4 | 6 |
| Genes with heterozygous sites in $III^M$ males, but without a significant temperature effect on expression in $III^M$ males | 6 | 19 |

Of the 14 genes with significant GxT effects and the requisite SNPs to test for allele-specific expression, only eight have a significant effect of temperature on gene expression within $III^M$ males (Figure 3; Table 1). Of those eight genes, four have a pattern of allelic expression consistent with the differential expression between 18°C and 29°C within $III^M$ males. To determine a null expectation for this proportion, we tested for concordance between allele-specific expression in $III^M$ males and 18°C vs 29°C expression differences for genes on other chromosomes. We do not expect any relationship between allele-specific expression and 18°C vs 29°C differences for the other chromosomes. We find that eight out of 14 genes in the rest of the genome have a significant allele-specific expression consistent with the 18°C vs 29°C expression differences (Table 1). Therefore, there is not an excess of genes on the third chromosome whose temperature-dependent expression is consistent with changes in

allele-specific expression (i.e., 4/8 < 8/14). This suggests that the GxT effects on expression of genes on the third chromosome is not the result of an excess of *cis*-regulatory differences between the $III^M$ and standard third chromosomes.

We could not do a similar analysis for head expression or for genes on the X vs $Y^M$ chromosomes because of small sample sizes of differentially expressed genes. We found only 1 gene on the third chromosome with a significant GxT interaction in head expression that also had at least one SNP in $III^M$ males and none at that variable site in $Y^M$ males. However, this gene did not have a significant effect of temperature on gene expression within $III^M$ males. In addition, there are only 40 genes assigned to the house fly X or $Y^M$ chromosome (R. P. Meisel & Scott, 2018), none of which have a significant GxT interaction affecting expression in testis (Supplementary Table 3). Only one X or $Y^M$ chromosome gene has a significant GxT interaction affecting expression in head (Supplementary Table 2), and it did not have any heterozygous sites.

## Discussion

We tested the hypothesis that GxT interactions affect gene expression in $Y^M$ and $III^M$ house fly males. These GxT effects could lead to differences in temperature-dependent phenotypes between house fly genotypes, thereby maintaining polygenic sex determination across latitudinal clines based on temperature-dependent fitness effects of the proto-Y chromosomes. To test this hypothesis, we used RNA-seq to compare gene expression in heads and testes of two nearly isogenic strains that differ only in their proto-Y chromosomes ($Y^M$ or $III^M$) that we raised at two different temperatures (18°C and 29°C). This 2x2 full factorial design allowed us to compare genome-wide expression between four GxT combinations, which we combined with targeted expression measurements of the male-determining gene (*Mdmd*) using qRT-PCR. We found that GxT interactions lead to differential gene expression in both head and testis, but the expression of genes involved in the sex determination pathway is not meaningfully affected by those GxT interactions.

### *No evidence that GxT interactions affect the sex determination pathway in a way that explains the maintenance of polygenic sex determination*

Evolutionary transitions between heritable and temperature-dependent sex determination systems are possible if sex determination pathways are temperature sensitive (Holleley et al., 2015; Quinn et al., 2007; Radder et al., 2008; Shine et al., 2002). Sex determination in flies operates by alternative splicing of multiple genes in the pathway (Bopp et

al., 2014; Salz, 2011). Temperature dependent alternative splicing has been reported in *Arabidopsis* (Steffen & Staiger, 2017; Streitner et al., 2013), *Neurospora* (Colot, Loros, & Dunlap, 2005), *Drosophila* (Jakšić & Schlötterer, 2016; Martin Anduaga et al., 2019), and mammals (Preußner et al., 2017). It is therefore possible that temperature-sensitive expression or splicing of sex determination factors can establish a clinal distribution of sex determination genes, such as what is observed in house fly (Schenkel et al. *in prep*). We tested if temperature-dependent expression of house fly sex determining genes is consistent with the geographical distributions of the $Y^M$ and $III^M$ proto-Y chromosomes. We did not find evidence for GxT interactions affecting the expression of the male-determining *Mdmd* gene or splicing of *Md-tra* in a way that are consistent with the clinal distribution of $Y^M$ and $III^M$ (Supplementary Figures 1 and 2). In addition, the expression of *Md-dsx*, an immediate downstream target of *Md-tra*, does not depend on GxT interactions (Supplementary Figure 3).

Our results suggest that GxT interactions affecting the sex determination pathway in house fly do not explain the maintenance of polygenic sex determination. Instead, other alleles present on either the $III^M$ chromosome or the $Y^M$ chromosomes may be the targets of selection. However, it is also possible that temperature affects the expression or splicing of sex determination pathway genes earlier in development than we measured. For example, *Mdmd* expression level might be more critical during early embryogenesis when *Md-tra* needs to be locked into a male or female mode of splicing. Hediger et al. (2010) have shown that the *Md-tra* auto-regulatory loop can be effectively shut down in embryos by RNAi, and male development proceeds normally without the need of *Mdmd* expression. Similarly, when *Mdmd* was removed from *Mdmd*/+ cells at embryonic stages, the resulting clones developed as males despite their female genotype (Hilfiker-Kleiner, Dübendorfer, Hilfiker, & Nöthiger, 1993). Thus the adult *Mdmd* and *Md-tra* expression we observed might not reflect the critical early expression levels. Additional work is required to further examine temperature-dependent effects on the expression or splicing of *Mdmd* or *Md-tra* across male genotypes in embryos, larvae, or pupae, rather than in adults.

Even though we did not observe *Mdmd* expression that is consistent with our hypothesis for the clinal distribution of $Y^M$ and $III^M$ males, we believe that an increased expression of *Mdmd* in $III^M$ males at the lower temperature (Supplementary Figure 1) is intriguing. It is possible that *Mdmd* expression is optimal at an intermediate level between high and low extremes: lower expression of *Mdmd* might be insufficient for *Md-tra* splicing, whereas higher expression of *Mdmd* might be toxic because of its proposed role in antagonizing functions of the generic

splicing factor *Md-ncm* (Sharma et al., 2017). The increased expression of *Mdmd* in $III^M$ males at a colder temperature (Supplementary Figure 1) might thus explain the absence of $III^M$ males in northern latitudes. Moreover, Hediger et al. (1998) found male determining regions on both arms of the $Y^M$ chromosome that act additively. However, it is not yet resolved whether *Mdmd* is the male determining factor on both of these arms or only one arm (Sharma et al., 2017). Additional work is required to determine if there is an additional male determining gene other than *Mdmd* on the $Y^M$ chromosome that may have temperature dependent activity.

***Temperature-dependent gene expression is not the result of large-scale cis-regulatory changes on the $III^M$ chromosome***

Previous RNA-seq experiments (Meisel et al., 2015; Son et al., 2019), as well as the results presented here (Figure 2A), provide consistent evidence that the third chromosome is enriched for genes that are differentially expressed between $Y^M$ and $III^M$ males. This is expected as the comparisons are between flies that differ in their third chromosome genotypes, and it suggests there are *cis*-regulatory effects on the expression of genes on the third chromosome. In contrast, we find that genes that are differentially expressed because of temperature are not enriched on the third chromosome in $III^M$ males (Figure 2B). We also found that GxT interactions affect the expression of 50 genes in head and 247 genes in testis, and these genes are not enriched on the third chromosome either (Supplementary Figure 9).

The lack of an enrichment of genes with temperature-dependent expression on the third chromosome suggests that temperature-dependent effects of the $III^M$ chromosome are not mediated by large-scale *cis*-regulatory changes across the $III^M$ chromosome. Consistent with this interpretation, we do not observe an enrichment of allele-specific expression in third chromosome genes that would be consistent with *cis*-regulatory effects driving temperature-dependent expression differences in $III^M$ males (Figure 3, Table 1). An independent analysis of a different RNA-seq data also found that there is not an excess of expression differences between $III^M$ and III alleles in a different house fly strain (Son & Meisel, 2020). We cannot perform a similar statistical analysis of $Y^M$ genes because of the small number of genes on that chromosome.

***Temperature-dependent gene expression and the maintenance of polygenic sex determination in house fly***

We hypothesized that targets of selection responsible for the maintenance of polygenic sex determination in house fly could be differentially expressed across proto-Y chromosome

genotypes and developmental temperatures. Despite our conclusion that a large number of *cis*-regulatory variants on the $III^M$ chromosome cannot explain the effect of the $III^M$ chromosome on temperature-dependent phenotypes, we still found evidence for temperature-dependent effects of the $III^M$ and $Y^M$ chromosomes that could explain their divergent phenotypic effects. First, there is some clustering by GxT combinations in the transcriptome-wide gene expression profiles. However, these differences seem to be mostly driven by differences in genotype rather than temperature, and the GxT clustering is only present in testis, not head (Figure 1 and Supplemental Figures 4-8). Second, we identify substantial temperature-dependent gene expression (Figure 2B) and many genes whose expression depends on GxT interactions (Supplementary Figure 9). These temperature-dependent effects on expression could be responsible for phenotypic differences between $Y^M$ and $III^M$ males, which could in turn provide a substrate upon which selection acts to maintain the $Y^M$-$III^M$ clines. If wide-spread *cis*-regulatory differences across proto-sex chromosomes are not responsible for these GxT effects (as we hypothesize above), then it is possible that a small number of loci on the proto-Y chromosomes act as temperature-dependent *trans* regulators of gene expression across the entire genome.

We also found multiple lines of evidence that reproductive traits are affected by the proto-Y chromosomes more than non-reproductive traits. First, we found a higher number of genes differentially expressed in testis because of GxT interactions than in head (Supplementary Figure 9). This is consistent with previous work that identified more differentially expressed genes in testis than head between $Y^M$ and $III^M$ males (Meisel et al., 2015). Second, genes associated with reproductive functions (as well as those associated with metabolism, development, and immune responses) were found amongst genes with significant GxT effects on expression. Genes responsible for male reproductive traits are more likely to be differentially expressed than other genes between males with different genotypes, possibly because of sexual selection (Meiklejohn, Parsch, Ranz, & Hartl, 2003). It is therefore possible that selection along the $Y^M$-$III^M$ cline acts on reproductive traits, or that the strength of sexual selection varies across the cline (Allen, Bonduriansky, Sgro, & Chenoweth, 2017; Arnqvist, 1992; Blanckenhorn, Stillwell, Young, Fox, & Ashton, 2006; Connallon, 2015; Payne & Krakauer, 1997). These traits, or other variants under selection, could have sexually antagonistic fitness effects (i.e., opposing fitness effects in males and females) which may be temperature-sensitive. Sexually antagonism is one of the few selection pressures predicted to maintain polygenic sex determination (Rice, 1986; G. S. van Doorn & Kirkpatrick, 2007). Population genetic modeling also predicts that sexually antagonistic effects of $Y^M$ and $III^M$ can maintain polygenic sex determination within

house fly populations (Meisel et al., 2016). It is therefore worth pursuing if sexual conflict can maintain polygenic sex determination by acting on temperature-dependent gene expression differences between $Y^M$ and $III^M$ males.

## Conclusion

The clinal distribution of the house fly proto-Y chromosomes in natural populations hints at a possible GxT interaction involved in maintaining polygenic sex determination (Denholm et al., 1986; Feldmeyer et al., 2008; Hamm et al., 2005; Hiroyoshi, 1964; Kozielska et al., 2008; Mcdonald et al., 1975). We did not find evidence that temperature-dependent expression or splicing of genes in the sex determination pathway explain the maintenance of polygenic sex determination in house fly. However, such effects may act earlier in development than we assayed. In contrast, GxT interactions affect gene expression in both somatic and reproductive tissues across the entire genome. Our results therefore suggest that alleles on the proto-Y chromosomes other than the male-determining *Mdmd* gene are targets of selection responsible for maintaining polygenic sex determination in house fly.

There is not an enrichment of GxT effects on the proto-Y chromosomes, suggesting that temperature-dependent expression differences between $Y^M$ and $III^M$ males (and thereby phenotypic and fitness effects of the proto-Y chromosomes) are not driven by a large-number of *cis*-regulatory changes on the $III^M$ chromosome. Instead, if temperature-dependent gene expression is responsible for temperature-dependent phenotypic effects of the $III^M$ and $Y^M$ proto-Y chromosomes, those effects are the result of a small number of alleles on the $III^M$ (and possibly $Y^M$) chromosome. Those genes would then have *trans* effects on gene expression across the entire genome. This is consistent with previous work that identified very few differentially expressed genes as a result of differences in proto-Y chromosome genotypes (Son et al., 2019; Son & Meisel, 2020). Therefore, we hypothesize that, if selection acts upon genome-wide temperature-dependent gene expression differences between $Y^M$ and $III^M$ males to maintain polygenic sex determination in house fly, there is a small number of alleles on the $Y^M$ or $III^M$ chromosomes that are responsible for those gene expression differences.

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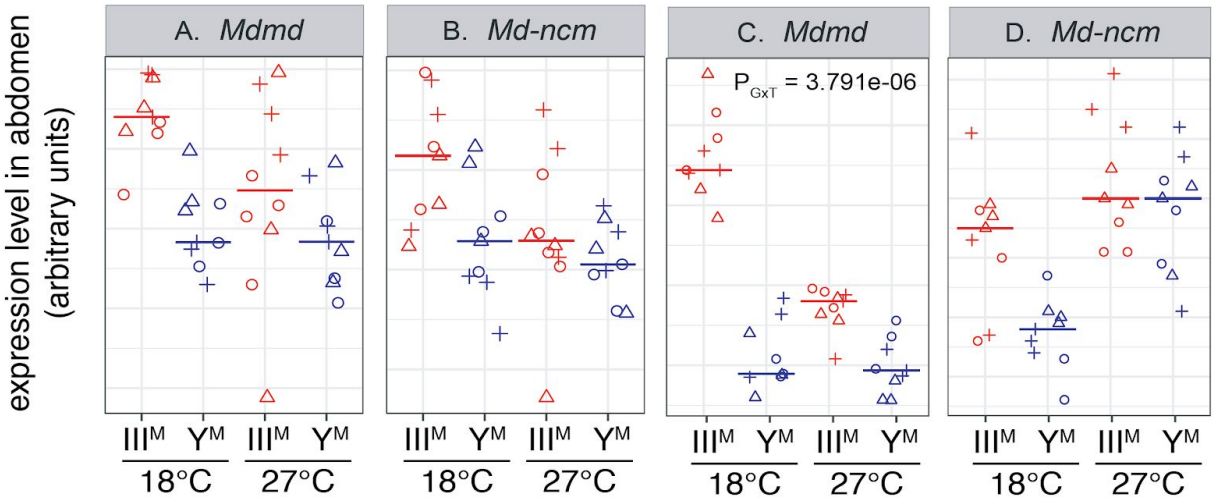
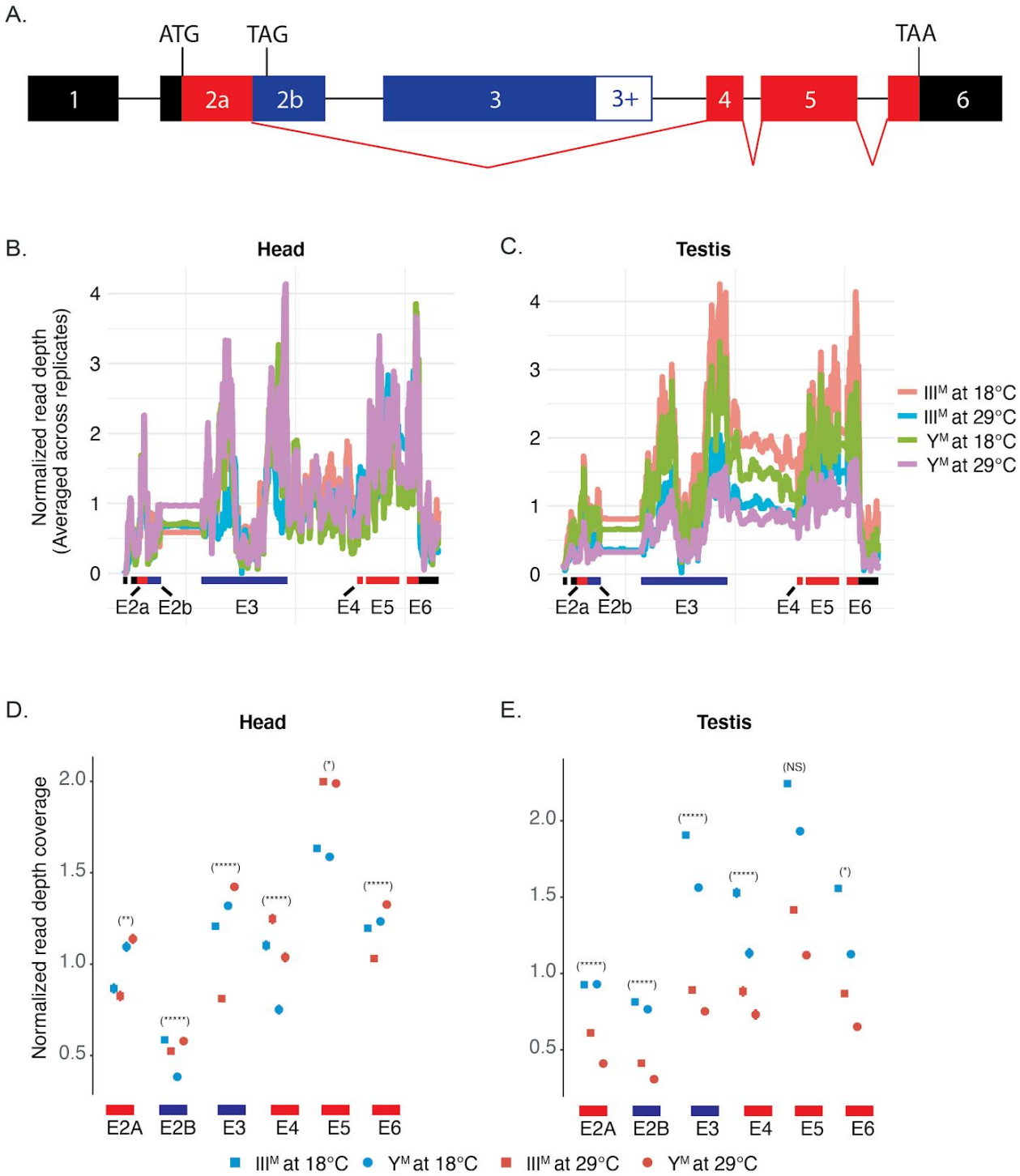
**Supplementary Figures**

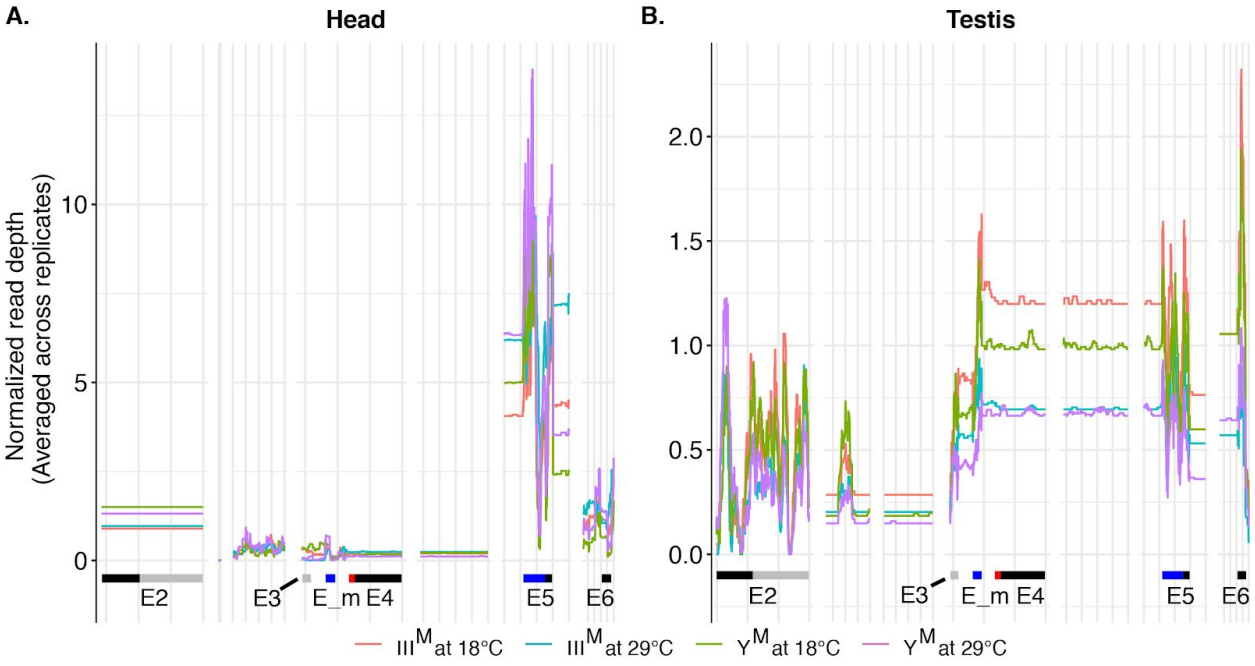



**Supplementary Figure 1.** Expression levels of *Mdmd* (A), and *Md-ncm* (B) in the abdomens of CSkab ($III^M$) and IsoCS ($Y^M$), and expression levels of *Mdmd* (C), and *Md-ncm* (D) in the whole body of SPA3 ($III^M$) and GK ($Y^M$) males raised at 18°C and 27°C. Each data point is a technical replicate, and points with the same shape are from the same biological replicate. The horizontal line indicates the median across all replicates.

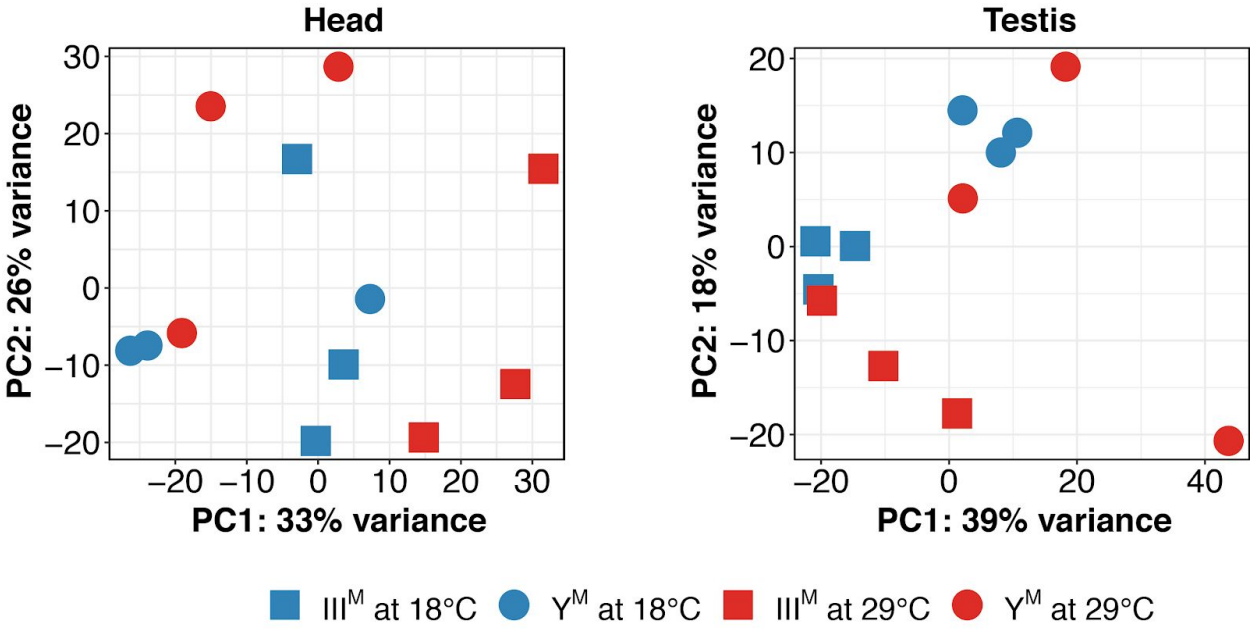



**Supplementary Figure 2.** (A) Schematic representation of the *Md-tra* locus based on DNA sequencing, cDNA clones, and RNA-Seq data (Monika Hediger et al., 2010; Jeffrey G. Scott et al., 2014). Splicing of the female-determining transcript is illustrated by the red diagonal lines connecting exons, and exons that contain protein-coding sequence of the female-determining splice variant are in red. Exons found in the male isoforms are shown in

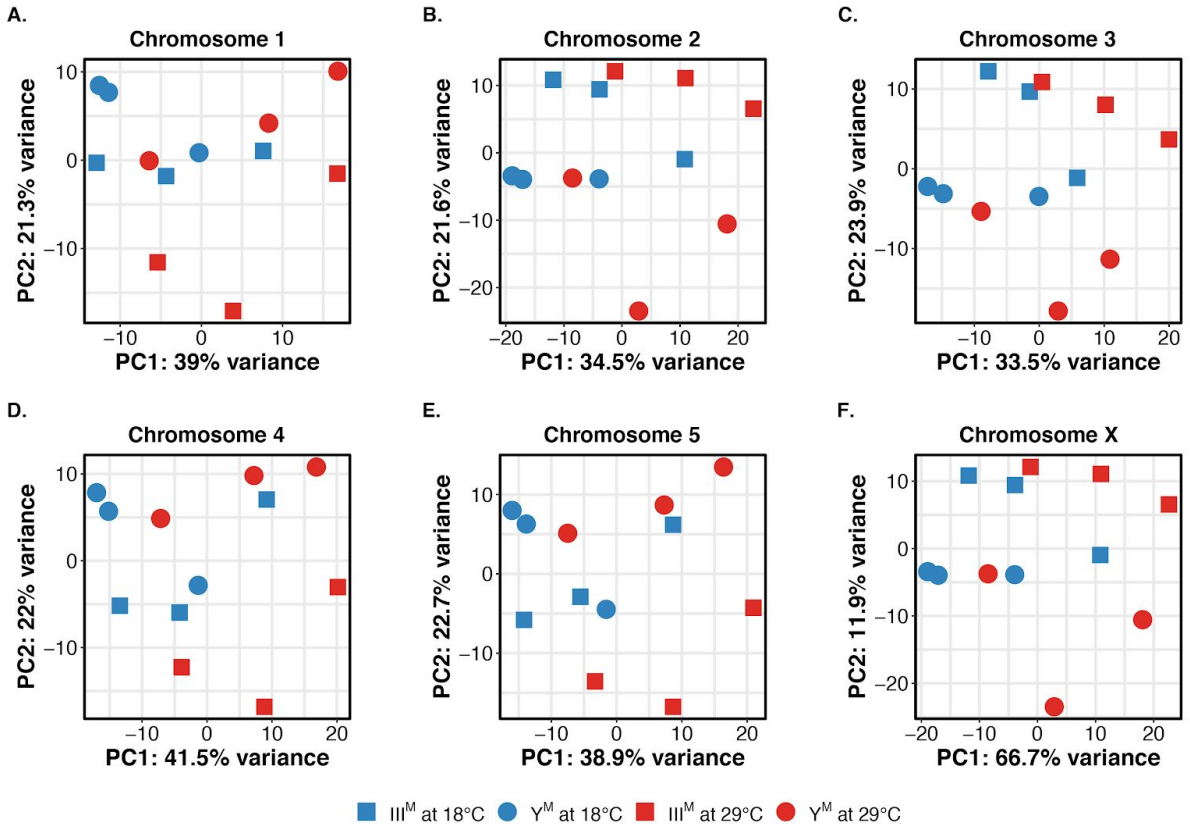
blue. The start and stop codon locations are shown.The expression of *Md-tra* across all exons is shown for RNA-seq data collected from head (B and D) and testis (C and E). Error bars represent standard error (most standard error estimates are smaller than the size of the points, and thus cannot be seen in the graph). Asterisks indicate *P* values obtained by comparing two linear models with and without GxT interaction terms with ANOVA using lmer () function in R (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, ****$P < 0.00005$, *****$P < 0.000005$).



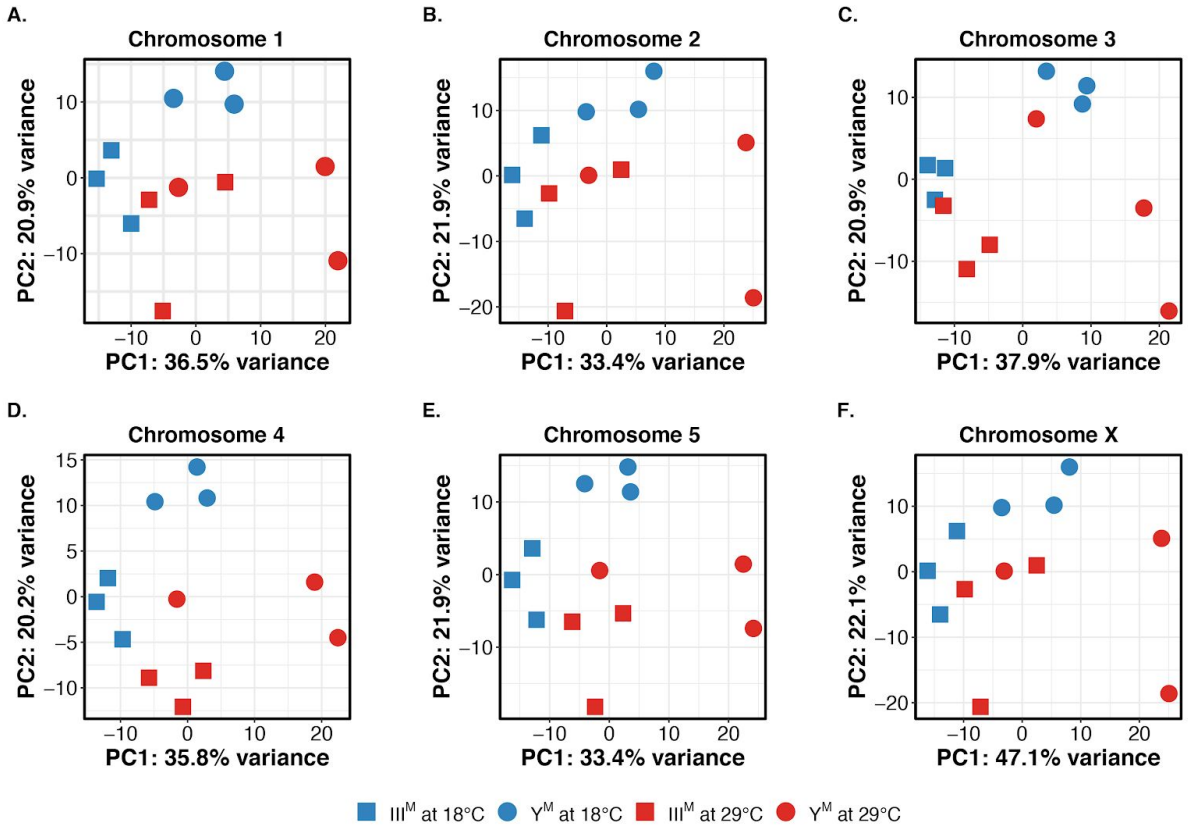

**Supplementary Figure 3.** Read depth coverage of *Md-dsx* in head (A) and testis (B) in each GxT combination. Exons are shown along the x-axis. Exons in the male-determining isoform are shown in blue, and exons in the female-determining isoform are shown in red. Expression of *Md-dsx* is not significantly affected by GxT interactions in either head or testis (Supplementary Tables 2 and 3). Usage of male-specific exon E_m (ANOVA, $P = 0.0004$), exon E5 (ANOVA, $P = 0.013$), and the female specific exon E4 (ANOVA, $P < 2.2e\text{-}16$) are affected by GxT interactions in testis. However, these GxT interactions are not in the directions expected if mis-splicing of *Md-dsx* is responsible for maintaining $Y^M$-$III^M$ clines. In head, usage of E_m (ANOVA, $P < 2.2$ e-16) and Exon 4 (ANOVA, $P < 2.2$ e-16) is affected by GxT interactions. Similar to testis, effects of the GxT interactions in head are also not in the directions expected if mis-splicing of *Md-dsx* is responsible for maintaining $Y^M$-$III^M$ clines.



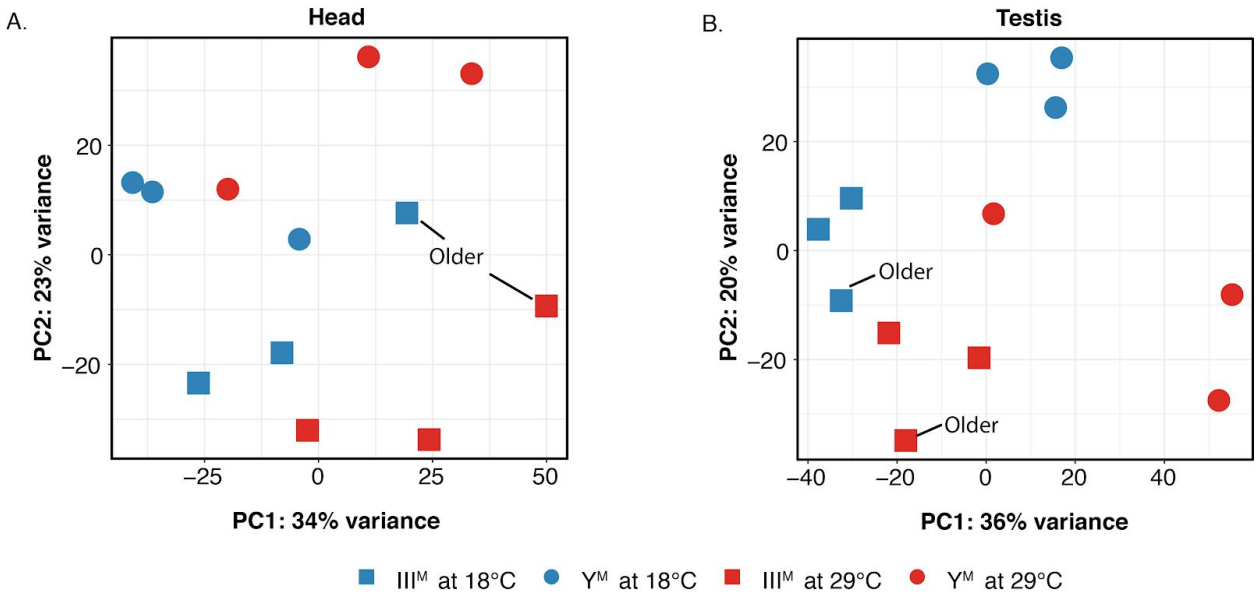

**Supplementary Figure 4.** PCA of the 500 most variable genes in both head and testis.



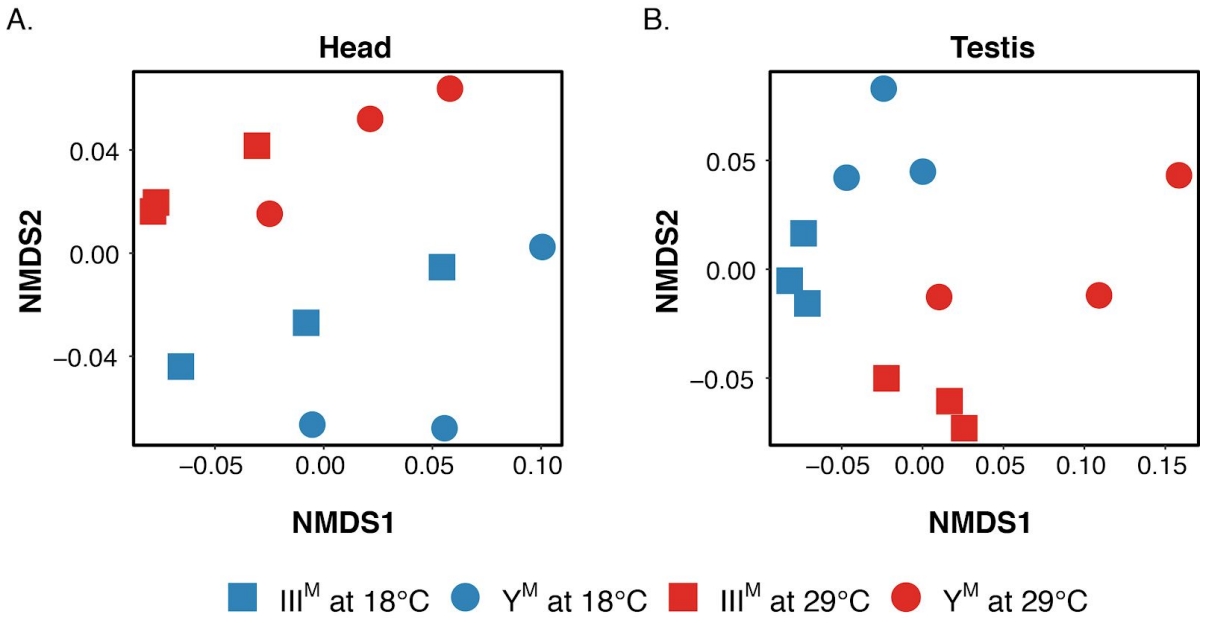

**Supplementary Figure 5.** PCA plots of gene expression levels in head on Chromosome 1 (A) Chromosome 2 (B) Chromosome 3 (C) Chromosome 4 (D) Chromosome 5 (E) and Chromosome X (F).



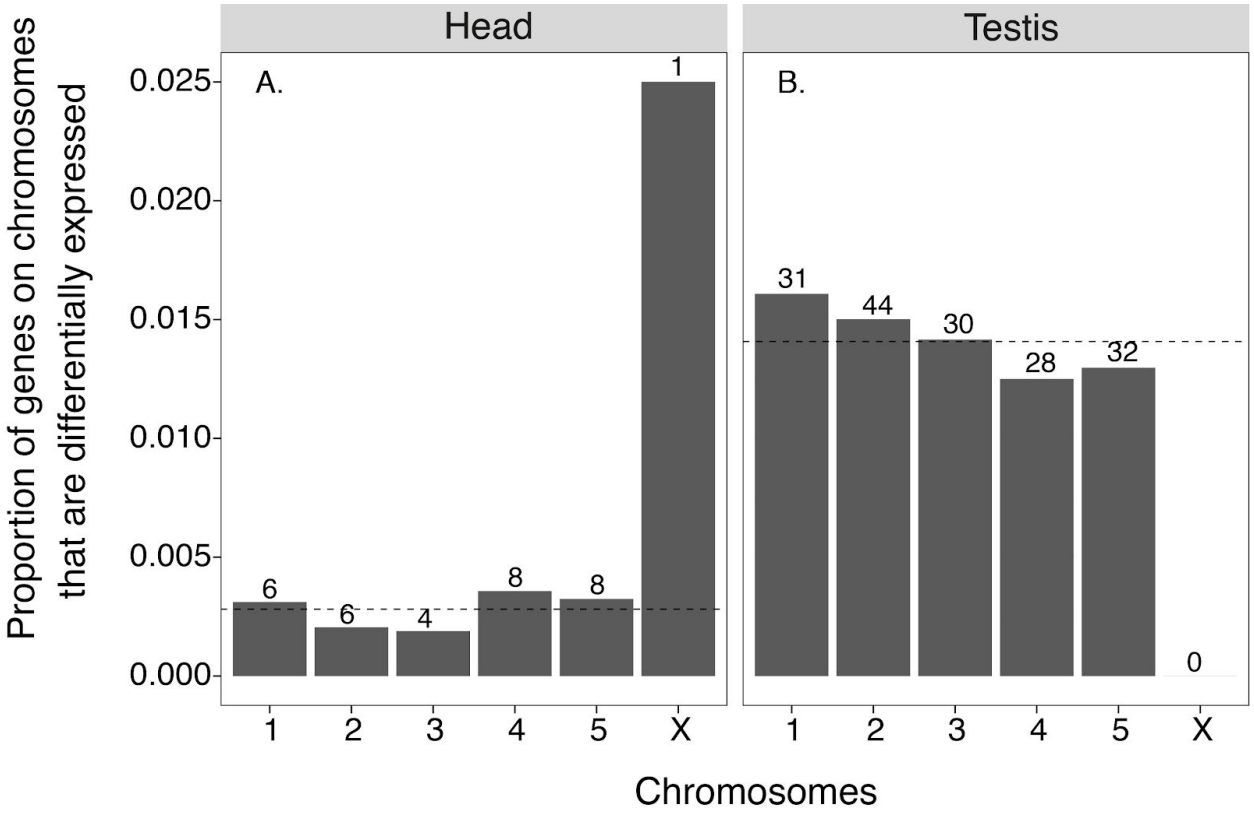

**Supplementary Figure 6.** PCA plots of gene expression levels in testis on Chromosome 1 (A) Chromosome 2 (B) Chromosome 3 (C) Chromosome 4 (D) Chromosome 5 (E) and Chromosome X (F).




**Supplementary Figure 7.** Tissue samples collected from 4-8 days old males have similar gene expression profiles as younger individuals in head (A) and testis (B).




**Supplementary Figure 8.** Non-metric multidimensional scaling (NMDS) plot showing gene expression profiles in Head (A) and Testis (B). These results are consistent with the PCA results (Figure 1).




**Supplementary Figure 9.** No chromosomes were enriched for significantly differentially expressed genes as a result of GxT interactions in head (A) and testis (B). The dashed line represents the genome-wide proportion of significantly differentially expressed genes. The numbers above each bar represent the number of significantly differentially expressed genes on that chromosome. Not all genes with significant GxT effects on expression are assigned to chromosomes, and only genes assigned to chromosomes are plotted. The X chromosome has <100 genes, which is much less than the other chromosomes which have 1000s of genes. Therefore a single GxT interaction on the X chromosome appears as a large proportion of genes. However, the small total number of X chromosome genes means this large proportion is not a significant excess.

**Supplementary Tables**

Supplementary Table 1. Primers for qPCR

| Gene | Forward Primer (5'-3') | Reverse Primer (5'-3') | Anneal |
|---|---|---|---|
| *Mdmd* | TGGTGCGCCCT TCTTTAAAC | GTTGACGCGGAC AATCAACG | 55℃ |
| *Md-ncm* | TTCCGACTCTG AATCATCTGAC | GCACTCCTCATA ATCCAAACTG | 55℃ |

Supplementary Table 2 provided separately.

Supplementary Table 3 provided separately.