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Temperature-dependent effects of house fly proto-Y chromosomes on gene expression could be responsible for fitness differences that maintain polygenic sex determination

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Abstract

Sex determination, the developmental process by which sexually dimorphic phenotypes are established, evolves fast. Evolutionary turnover in a sex determination pathway may occur via selection on alleles that are genetically linked to a new master sex determining locus on a newly formed proto-sex chromosome. 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 and sex chromosomes. 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 two most common male-determining proto-Y chromosomes form latitudinal clines on multiple continents, suggesting that temperature variation is an important selection pressure responsible for maintaining polygenic sex determination in this species. Temperature-dependent fitness effects could be manifested through temperature-dependent gene expression differences across proto-Y chromosome genotypes. These gene expression differences may be the result of *cis* regulatory variants that affect the expression of genes on the proto-sex chromosomes, or *trans* effects of the proto-Y chromosomes on genes elswhere in the genome. We used RNA-seq to identify genes whose expression depends on proto-Y chromosome genotype and temperature in adult male house flies. We found no evidence for ecologically meaningful temperature-dependent expression differences of sex determining genes between male genotypes, but we were probably not sampling an appropriate developmental time-point to identify such effects. In contrast, we identified many other genes whose expression depends on the interaction between proto-Y chromosome genotype and temperature, including genes that encode proteins involved in reproduction, metabolism, lifespan, stress response, and immunity. Notably, genes with genotype-by-temperature interactions on expression were not enriched on the proto-sex chromosomes. Moreover, there was no evidence that temperature-dependent expression is driven by chromosome-wide *cis*-regulatory 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 that may have *trans* effects on the expression of genes on other chromosomes.

KEYWORDS
development and evolution, evolution of sex, genomics/proteomics, insects, transcriptomics

1 | 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 development 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). Most population genetics models predict that polygenic sex determination will be an evolutionary intermediate between different monogenic sex determination systems, and the factors responsible for maintaining polygenic sex determination as a stable polymorphism are poorly understood (van Doorn, 2014; Rice, 1986). Models that do allow for the stable maintenance of polygenic sex determination require opposing (sexually antagonistic) fitness effects of sex chromosomes in males and females (van Doorn & Kirkpatrick, 2007, 2010; Orzack et al., 1980). The sexually antagonistic fitness effects of the sex chromosomes are often hypothesized to be the result of alleles that are genetically linked to the sex determining locus, and there is some evidence in support of this hypothesis (Roberts et al., 2009). Understanding how selection acts on these alleles to maintain polygenic sex determination would provide valuable insight into the factors that drive the evolution of sex determination and sex chromosomes.

House fly (*Musca domestica*) is a well suited model for studying polygenic sex determination because multiple male and female determining loci segregate on different proto-sex chromosomes in natural populations (Hamm et al., 2015). Male sex in house fly is initiated by the gene *Musca domestica male determiner*, *Mdmd* (Sharma et al., 2017). *Mdmd* arose via the recent duplication of the ubiquitous splicing factor *nucampholin* (*Md-ncm*) after the divergence of house fly from its close relative *Stomoxys calcitrans*. *Mdmd* promotes male development by causing the house fly orthologue of *transformer* (*Md-tra*) to be spliced into nonfunctional isoforms with premature stop codons (Hediger et al., 2010). The lack of functional Md-Tra protein leads to male-specific splicing of *doublesex* (*Md-dsx*) and *fruitless* (*Md-fru*), the two known downstream targets of *Md-tra* (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). While Y^M is conventionally referred to as the Y chromosome, both III^M and Y^M are young proto-Y chromosomes that are minimally differentiated from their homologous proto-X chromosomes (Meisel et al., 2017; Son & Meisel, 2021). Each proto-Y chromosome (Y^M and III^M) has a dominant male-determining activity relative to its homologous proto-X chromosome (X and III, respectively). 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 et al., 1986; Hamm et al., 2005; Hiroyoshi, 1964; McDonald et al., 1975). The frequencies of III^M and Y^M in natural populations have remained stable for decades (Kozielska et al., 2008; Meisel et al., 2016). This clinal distribution of III^M and Y^M, along with their stable frequencies across populations, suggests that natural selection maintains the polymorphism.

A female determining allele of *Md-tra* (*Md-tra*^{D}) is also found in some house fly populations (Hediger et al., 2010; McDonald et al., 1978). *Md-tra*^{D} is a dominant allele with epistatic effects, and flies carrying a single copy of *Md-tra*^{D} develop as females even if they carry three *Mdmd* chromosomes (Hediger et al., 1998; Schmidt et al., 1997). In some populations, both Y^M and III^M can be found, with some males carrying one copy of two different proto-Y chromosomes or homozygous for a proto-Y (e.g., Hamm & Scott, 2008, 2009). *Md-tra*^{D} is most common in populations with a high frequency of these "multi-Y" males, which results in a sex ratio with an equal number of males and females (Meisel et al., 2016).

The natural distribution of III^M and Y^M hints at a possible genotype-by-temperature (G×T) interaction that could explain the stable maintenance of Y^M-III^M clines. Temperature is not the only selection pressure that could vary along the clines, but seasonality in temperature is the best predictor of the frequencies of the proto-Y chromosomes across populations (Feldmeyer et al., 2008). Moreover, Y^M and III^M affect thermal tolerance and preference in male house flies in a way that is consistent with their clinal distribution (Delclos et al., 2021). There are at least two nonexclusive ways in which temperature-dependent selection pressures could maintain the III^M-Y^M polymorphism. First, alleles on the III^M and Y^M chromosomes (other than the *Mdmd* locus) could have temperature-dependent phenotypic effects that affect fitness. Second, it is possible that the *Mdmd* loci on the III^M and Y^M chromosomes differ in their temperature-dependent activities, such that *Mdmd* on the III^M chromosome increases male fitness at warm temperatures and *Mdmd* on the Y^M chromosome increases fitness at colder temperatures. Such

temperature-dependent effects of sex determining genes has been observed in fish and reptiles, overriding the outcomes of sex determining genotypes (Holleley et al., 2015; Quinn et al., 2007; Radder et al., 2008; Shine et al., 2002).

We investigated if temperature-dependent phenotypic effects of III^M and Y^M could be caused by differential gene expression in males across temperatures. These temperature-dependent phenotypic differences between males carrying either III^M or Y^M could be responsible for fitness differences underlying their clinal distribution and the maintenance of polygenic sex determination. We selected gene expression as a phenotypic read-out of G × T interactions because temperature-dependent differences in gene expression are well documented in clinally distributed genetic variation (e.g., Levine et al., 2011; Zhao et al., 2015). We used RNA-seq to study gene expression in two nearly isogenic lines of house flies, differing only in whether they carry III^M or Y^M, reared at two developmental temperatures. This allowed us to assess the effects of the entire III^M and Y^M chromosomes (including the *Mdmd* loci and linked alleles) on gene expression throughout the genome. We used these data to identify genes whose expression depends on G × T interactions. We also tested if temperature-dependent expression differences between III^M and Y^M males can be explained by large-scale divergence of *cis*-regulatory alleles between the proto-Y chromosomes (III^M and Y^M) and their homologous proto-X chromosomes. We additionally used quantitative reverse transcription PCR (qRT-PCR) to investigate the temperature-dependent expression of *Mdmd*.

2 | MATERIALS AND METHODS

2.1 | qRT-PCR samples and analysis

We used qRT-PCR to measure the expression of *Mdmd* and its paralogue *Md-ncm* in two Y^M strains and two III^M strains. The strains were grouped into two pairs, with 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 (Scott et al., 1996). IsoCS was previously created by crossing a Y^M chromosome from Maine onto the CS background (Hamm et al., 2009). We created CSkab by backcrossing the III^M chromosome from the KS8S3 strain collected in Florida (Kaufman et al., 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). GK-1 and SPA3 were maintained in the laboratory, each as inbred populations, for approximately 40 and 50 generations, respectively.

We raised all strains at 18°C and 27°C for two generations with 12 h:12 h light:dark photoperiods. Adult males and females for each G × T combination were housed in cages with ad libitum containers of 1:1 combinations of sugar and nonfat 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 h (Hamm et al., 2009). The resulting larvae were maintained in the same media within 32 oz containers. Adult females did not lay a sufficient number of 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 anaesthetized 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 anaesthetized 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 1.5 ml 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 no. K1632) according to the manufacturer's protocol.

We conducted qRT-PCR of cDNA from the male flies. We used qRT-PCR primers (Table S1) 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 et al., 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 an 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 G × T 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 microtitre 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) from the serial dilutions 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 et al., 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 G × T interaction on the expression of the transcript.

2.2 | RNA-seq samples

We used RNA-seq to measure gene expression in the Y^M strain 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 (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). These strains are normally raised at 25°C, but were raised at different temperatures (18°C or 29°C) in our experiment in order to determine the effect of genotype and temperature on gene expression.

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 therefore had four combinations of genotype (Y^M and III^M) and temperature (18°C and 29°C). We controlled for the adult density using 35 adult males and 35 adult females for each G × T 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 anaesthetized with CO_2. We dissected testes from 15–20 house flies per each of three replicates of each G × T combination. Similarly, 5 heads were dissected for each of three biological replicates for each G × T 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 Bioanalyser (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 G × T 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).

2.3 | RNA-seq data analysis

RNA-seq reads were aligned to the annotated house fly reference genome Musca_domestica-2.0.2 (Scott et al., 2014) using HISAT2 (Kim et al., 2015) with the default settings of a maximum mismatch penalty of 6 and minimum penalty of 2, and a soft-clip penalty of maximum 2 and minimum 1 (Tables S2 and S3). Soft-clipping performs as well or better than read-trimming for removing adapters and other artifactual sequences from RNA-seq reads (Liao & Shi, 2020), while not introducing biases caused by read-trimming (Williams et al., 2016). 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 in HTSeq (Anders et al., 2015). We only included uniquely mapped reads, and we excluded reads with ambiguous mapping and reads with a mapping quality of less than 10.

We analysed the exon-level expression of the sex determining genes *Md-tra* (LOC101888218) and *Md-dsx* (LOC101895413) for each G × T combination. To do so, we first determined the read coverage across *Md-tra* and *Md-dsx* transcripts using the mpileup function in SAMtools (Li et al., 2009). We then calculated normalized read depth (D_{ijk}) at each site *i* within each gene in library *j* for each G × T 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 G × T combination ($\underline{D}_{ik}$).

We also used the DESeq2 package in R (Love et al., 2014) to analyse differential expression of all annotated genes between all G × T combinations. To do so, we used a linear model that included genotype (Y^M or III^M), developmental temperature, and their interaction term to predict gene expression levels:

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

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 G × T 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 analysed with the full model. For principal component analysis (PCA), hierarchical clustering, and nonmetric multidimensional scaling (NMDS), we analysed regularized log transformed count data generated by the rlog() function in DESeq2. NMDS was carried out using the metaMDS() function from the vegan package in R with the autotransform = FALSE option (Oksanen et al., 2019). A full list of commands and files used in the differential expression analysis are provided elsewhere (Meisel & Adhikari, 2021).

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 was used to assign a FlyBase ID to each house fly transcript. These *D. melanogaster* homologues were then used in DAVID 6.8 (Huang et al., 2009a, 2009b) to identify GO terms that are significantly enriched amongst differentially expressed genes (FDR corrected $p < .05$).

2.4 | 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 (non-*Mdmd*) third chromosome in III^M males. To do so, we 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; Meisel et al., 2017). 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 reduce read-mapping biases associated with an 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 (Meisel et al., 2017) to recalibrate the realigned reads. The realigned reads were then used for variant calling with HaplotypeCaller with emission and calling thresholds 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. This filtering was applied because there may be preferential mapping of reads containing SNPs found in the reference genome relative to reads with alternative SNPs (Stevenson et al., 2013; Zimmer et al., 2016). By excluding SNPs found in clusters of at least 3 in a 35 bp window from our analysis, we can greatly reduce read-mapping biases from our estimates of allele-specific expression (Son & Meisel, 2021).

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 G × T 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. A full list of commands and files for the allele-specific expression analysis are provided elsewhere (Meisel & Adhikari, 2021).

3 | RESULTS

3.1 | Genotype and temperature affect genome-wide gene expression profiles

We used RNA-seq to test for the effects of genotype and developmental temperature on gene expression in heads and testes of Y^M and III^M house fly males raised at 18°C and 29°C. The purpose of

raising the strains at two different temperatures was to expose G × T effects of Y^M and III^M alleles sampled from natural populations (i.e., genotype-dependent plasticity across temperatures), not to evolve adaptations to each temperature. We first used PCA, NMDS, and hierarchical clustering to assess the similarities of the overall gene expression profiles of each of three replicates of each G × T combination in head and testis separately.

The PCA of the head RNA-seq data (using all 16,540 expressed genes) provided some evidence for an effect of genotype on gene expression. The first principal component (PC1) of head gene expression explained 34% of the variance in expression, and the second (PC2) explained 23% of the variation (Figure 1a). However, there was no clear grouping by genotype or developmental temperature, which can be best explained by an age-effect in our samples. One biological replicate of III^M heads at each temperature came from older males (4–8 days old, as opposed to the other samples which were 1–3 days old). The two older samples had head expression profiles that clustered separately from the remaining samples in our PCA (Figure 1a). Excluding the two older samples, there was a clear grouping by genotype along PC2, which explained 28% of the variance in head gene expression (Figure 1b). Because of the effect of age on head gene expression, we describe results both including and excluding the two older samples in the remainder of the analyses we present.

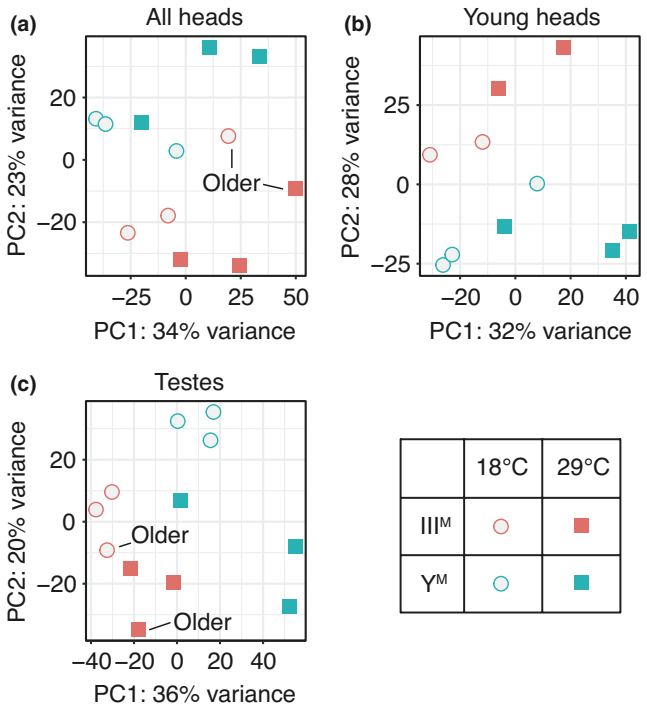

FIGURE 1 Effect of genotype and temperature on genome-wide gene expression in house flies. Graphs show the first two principal components (PC) explaining gene expression levels in all male heads (a), heads of young males only (b), and testes (c). Each data point represents a biological replicate, with PC coordinates determined using regularized log transformed read counts

In testis, PC1 explained 36% of the variance in expression, and it separated III^M males at 18°C from Y^M males at 29°C (Figure 1c). III^M is found at southern, warmer temperatures, whereas Y^M is found at northern, colder temperatures. PC1 for testis expression therefore separated the two genotypes at the temperatures that are opposite from their geographic distribution (i.e., Y^M occurs at relatively low temperature and III^M at high temperature). PC2 explained 20% of the variation in testis expression, and it separated III^M at 29°C from Y^M at 18°C (Figure 1c). Therefore, PC2 separated the two genotypes at temperatures that are consistent with their geographic distribution. There was not a meaningful effect of age on gene expression in testis (Figure 1c), and we thus did not repeat the analysis excluding the older testis samples.

We performed the following analyses to evaluate the robustness of our PCA results. First, we carried out PCA by considering only the 500 most variable genes in head and testis, and we observed the same patterns as those described above (Figure S1). We additionally carried out PCA for genes on each chromosome, and the results for each chromosome were consistent with those across all chromosomes (Figures S2–S4). Notably, there was very strong differentiation of III^M and Y^M males when we considered the testis expression of X chromosome and third chromosome genes (Figure S4). This can be explained by the fact that the two genotypes only differ in these chromosomes, and they share the same genetic background for the remaining chromosomes. We also carried out NMDS and hierarchical clustering of the RNA-seq data. There was grouping by genotype in the NMDS for the head samples, and grouping by genotype and temperature in the testis samples (Figure S5). In the hierarchical clustering, there was no grouping by genotype or temperature in head samples while including or excluding the older samples (Figure S6). For testis gene expression, there was some evidence for clustering first by genotype and then by temperature (Figure S6), similar to the PCA. However, the concordance between clusters and G × T combinations was not perfect.

3.2 | Genotype and temperature affect the expression of individual genes

To further test for genotype- and temperature-dependent gene expression, we next identified differentially expressed genes in two types of pairwise comparisons: (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 genotypes, there were 900 genes 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 (Table S4, Figure S7). Excluding the two older samples, 786 genes were differentially expressed between Y^M and III^M heads at 18°C, and 1748 genes were differentially expressed between Y^M and III^M heads at 29°C (Table S5, Figure S7). The increase in differentially expressed genes at 29°C when the older samples were excluded can be explained by reduced variation within the III^M male samples, which increased our power to

detect differences between III^M and Y^M males. The number of differentially expressed genes was higher in testis than head: 2413 genes at 18°C and 2199 genes at 29°C were significantly differentially expressed between Y^M and III^M testes (Table S6, Figure S7). 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).

When comparing between temperatures within each genotype, there were 739 genes significantly differentially expressed between heads of Y^M flies raised at the two different temperatures (Table S4, Figure S7). Similarly, 744 genes were differentially expressed between the heads of III^M flies raised at different temperatures (Table S4, Figure S7). When we excluded older samples, we found 828 genes differentially expressed between heads of III^M males raised at different temperatures and 1280 genes differentially expressed between the heads of Y^M males (Table S5, Figure S7). Once again, the increase in differentially expressed genes when the older samples were excluded can be explained by greater power to detect differential expression when the outlier III^M males were removed. This also increased our power to detect differences within Y^M males because we analysed the data with a statistical model that included all genotypes, temperatures, and their interactions. In testis, there were 2402 genes in Y^M flies and 1649 genes in III^M flies that were differentially expressed between 18°C and 29°C (Table S6, Figure S7).

In both tissue samples and temperatures, an excess of genes on the third chromosome were differentially expressed between Y^M and III^M males (Figure 2a), regardless of whether the older samples were excluded (Figure S8). This is consistent with previous comparisons of Y^M and III^M males (Meisel et al., 2015; Son et al., 2019). In contrast, there is no significant chromosomal enrichment of genes that were differentially expressed between temperatures in either head or testis when we included all samples (Figure 2b). However, there is a modest enrichment of third chromosome genes that were differentially expressed between temperatures in young Y^M heads, that is, excluding the two older samples (Figure S8). This is surprising because all Y^M males should have the same third chromosome genotype, and we do not have an explanation for this pattern. The observed proportion of differentially expressed X-linked genes also appears to deviate from the expectation based on the genome-wide average (Figure 2a), but it is not significant because of low power caused by the small number (<100) of genes on the house fly X chromosome (Meisel & Scott, 2018).

3.3 | G × T interactions affect the expression of a small subset of genes

We next identified individual genes that were 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. There were 50 genes in head and 247 genes in testis whose expression significantly differed in response to the G × T interaction when we included all samples (Tables S4 and S6, Figure S7). In comparison, 108 genes were differentially expressed in heads in response to the G × T interaction when the two older samples were excluded (Table S5, Figure S7). Of the genes for which the G × T interaction significantly affected expression in head, 26 genes are shared by the analysis of all heads and when the two older samples were excluded (Figure S9). There is not an enrichment of genes with

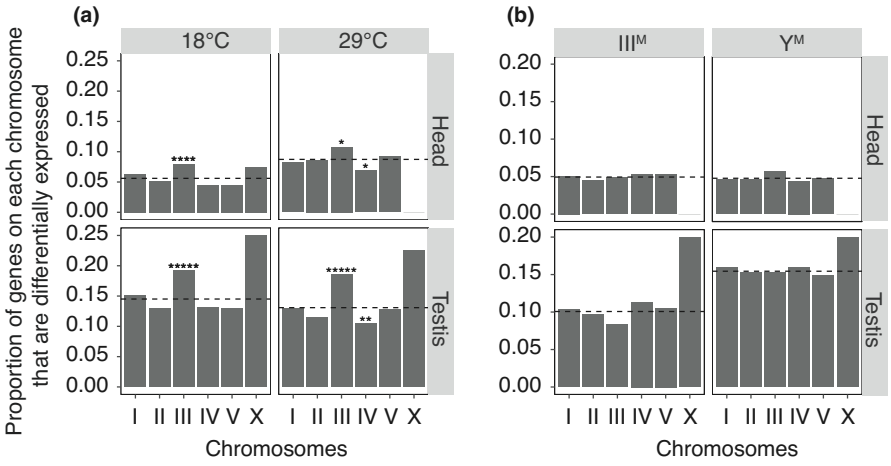

FIGURE 2 Genes that are differentially expressed between house fly genotypes are significantly enriched on the third chromosome. (a) The proportion of house fly genes on each chromosome that were differentially expressed (DE) between Y^M and III^M males is plotted for heads (top) and testes (bottom) of flies raised at 18°C (left) or 29°C (right). (b) The proportion of house fly genes on each chromosome that were DE between temperatures is plotted for heads (top row) and testes (bottom row) for III^M (left) and Y^M (right) males separately. Each bar represents the proportion of DE genes on a chromosome (number of DE genes / number of genes on the chromosome), and dashed lines show the the proportion of DE genes across the genome (number of DE genes/number of genes assigned to any chromosome). Asterisks indicate *p*-values obtained from Fisher's exact test comparing the number of DE genes on a chromosome, the number of non-DE genes on a chromosome, and the number of DE and non-DE genes across all other chromosomes, after Bonferroni correction (*$p < .05$, **$p < .005$, ***$p < .0005$, ****$p < .00005$, *****$p < .000005$)

significant G × T interactions on any chromosome in all male heads, younger male heads, or testes (Figure S10).

There were 10 genes whose expression was affected by G × T interactions in both head and testis (Figure S9). We would expect <1 gene to be affected by G × T interactions in both head and testis if the G × T effects were independent across tissues. The 10 genes we observed are significantly greater than this expectation ($z = 10.12$, $p < 2.2 \times 10^{-16}$, in a test of proportions), suggesting G × T effects on expression are not independent across tissues. Similarly, nine genes were affected by G × T interactions in both testis and young male heads (Figure S9), which is significantly greater than the expectation of <2 genes ($z = 5.42$, $p = 5.9 \times 10^{-8}$, in a test of proportions). An excess of genes were also differentially expressed in both head and testis in all pairwise comparisons between genotypes and temperatures (Figure S9). A similar nonindependence of expression differences across tissues was previously observed between Y^M and III^M males (Meisel et al., 2015).

We characterized the functional annotations of genes that were differentially expressed as a result of G × T interactions. We did not find any GO terms associated with genes significantly differentially expressed as a result of G × T interactions in either testis or head, regardless of whether we included all head samples or excluded the two older samples. However, individual genes are suggestive of biological functions that could be affected by G × T interactions on expression. In head, the genes that were differentially expressed because of G × T interactions include an apolipoprotein-D gene (*LOC101893129*). This gene is homologous to *D. melanogaster NLaz*, which is involved in stress response (Hull-Thompson et al., 2009), and it was upregulated in III^M males at 29°C (Figure 3a). Two genes encoding immune effectors (*LOC105261620*, which encodes a defensin; and *LOC101895951*, which encodes a lysozyme and is homologous to *D. melanogaster LysP*) were also upregulated in III^M at 29°C (Figure 3a). Three DNA repair genes (*LOC101889156*, homologous to *D. melanogaster Gen*, encoding XPG-like endonuclease; *LOC101899772*, homologous to *maternal haploid*, *mh*, which encodes a protease; and *LOC101899952*, homologous to *Stromalin*, *SA*) are upregulated in Y^M at 18°C (Figure 3a). Lastly, an odorant binding protein-coding gene (*LOC105261913*, homologous to *D. melanogaster Obp56h*) was upregulated in Y^M males at 29°C (Figure 3a).

We also identified genes whose expression depended on the G × T interaction when we excluded the two older head samples. *LOC101895951* (*LysP*), *LOC105261913* (*Obp56h*), *LOC101889156* (*Gen*), *LOC101899772* (*mh*), and *LOC101899952* (*SA*) were also significantly differentially expressed in younger heads in the same direction as when we analyse all male head samples (Figure S15). A similar pattern was observed for *Nlaz* expression when we only included young heads, although the G × T effect was not significant (Figure S15). Four other genes only had significant G × T effects in young male heads, including three genes related to muscle performance (*LOC101893720*, homologous to *D. melanogaster bent*, *bt*; *LOC101895658*, homologous to *Unc-89*; and *LOC101901052*, homologous to *Myofilin*, *Mf*), which were all upregulated in Y^M males at 18°C (Figure 3b). One gene involved in endoplasmic reticulum (ER) stress response (*LOC101901283*, homologous to *Calx*) was upregulated in III^M males at 29°C (Figure 3b).

In testis, genes with significant G × T effects on expression included those coding for proteins related to reproductive functions: the protamine *ProtB* homologue *LOC101887804*; the *asunder* (*asun*) homologue *LOC101899763*; the *sarah* (*sra*) homologue *LOC101894442*, and the *Farnesyl pyrophosphate synthase* (*Fpps*) homologue *LOC101896699* (Figure 4). Other notable genes that were differentially expressed in testis because of G × T interactions include three metabolic genes (*LOC109613297*, which encodes a hexokinase and is homologous to *D. melanogaster Hex-t2*; *LOC101901027*, which encodes fructose-1,6-bisphosphatase and is homologous to *D. melanogaster fbp*; and *LOC101901154*, which encodes an aldehyde oxidase, homologous to *AOX3*), all of which were upregulated in Y^M males at 18°C (Figure 4). One adult lifespan related gene (*LOC101897626*, the homologue of *D. melanogaster pointed*, *pnt*) was downregulated in III^M males at 29°C, and another lifespan related gene (*LOC101897352*, which encodes cystathionine β-synthase, *Cbs*) was upregulated in Y^M males at 18°C (Figure 4). Lastly, two immunity-related genes were differentially expressed in testis. One of the immune genes (*LOC101887442*, which encodes a Gram-negative bacteria-binding protein and is homologous to *GNPB3*) was upregulated in Y^M males at 18°C, and the other (*LOC101895929*, which is homologous to *D. melanogaster Phenoloxidase 1*, *PPO1*) was upregulated in III^M males at 18°C (Figure 4).

3.4 | G × T interactions affecting expression of genes in the sex determination pathway

We did not find evidence that the sex determining gene *Md-tra* was differentially expressed according to a G × T interaction in either all male heads (Table S4), young male heads (Table S5), or testes (Table S6). However, G × T interactions affected the expression of most *Md-tra* exons in heads (including or excluding older samples) and testes (Figures S11 and S12). If a G × T interaction affecting the mis-splicing of *Md-tra* were responsible for the Y^M–III^M cline, we would expect more female-determining isoforms produced (i.e., misexpressed) at temperatures that are discordant with the distribution of each proto-Y chromosome (misexpression of female-determining isoforms in Y^M males raised at a high temperature, or III^M males raised at a low temperature). In contrast to that expectation, the G × T interactions were not in the directions consistent with mis-splicing of *Md-tra* at discordant temperatures (Figure S11 and S12). 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 G × T 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 provide no evidence for an effect of G × T interactions in the expression of *Md-dsx* or *Md-fru* in all male heads (Table S4), young male heads (Table S5), or testes (Table S6). We also found no evidence of G ×

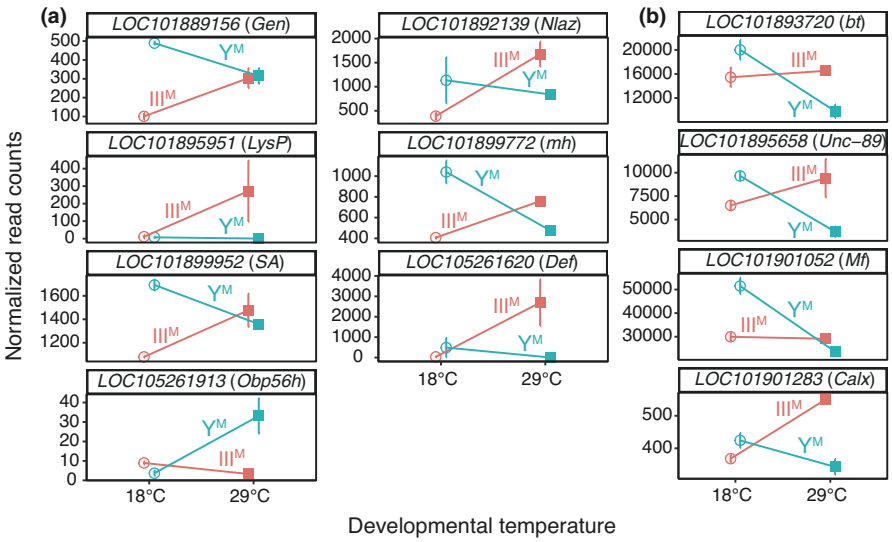

FIGURE 3 G × T interactions affect gene expression in house fly head. Graphs show normalized read counts (obtained using DESeq2) for genes significantly differentially expressed because of G × T interactions in (a) all male head samples or (b) young male heads only. Genes are identified based on their house fly gene ID followed by their *Drosophila melanogaster* homologues in parenthesis. Error bars represent standard errors of the mean

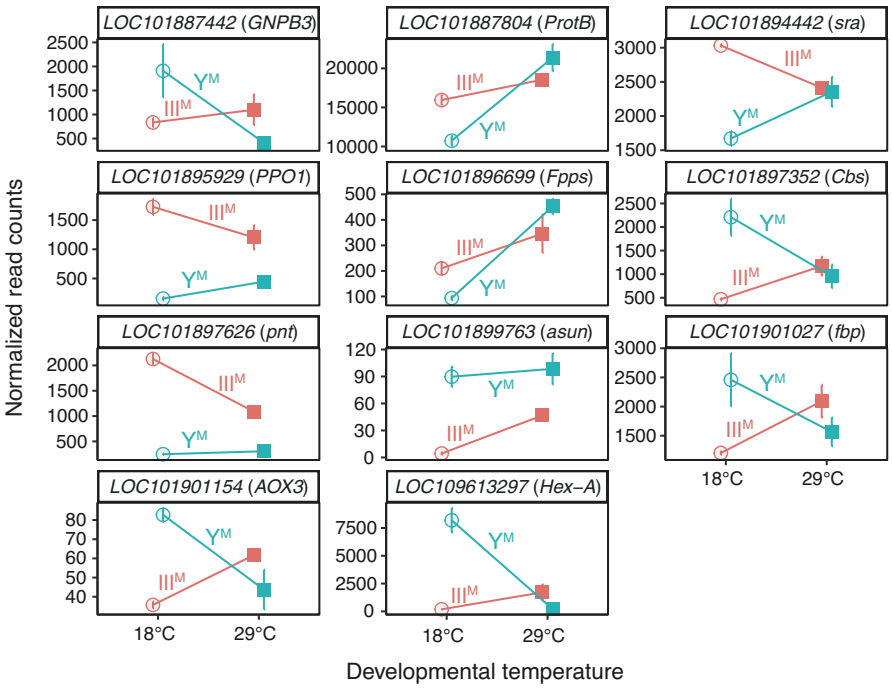

FIGURE 4 G × T interactions affect gene expression in house fly testis. Graphs show normalized read counts (obtained using DESeq2) for genes significantly differentially expressed in testis because of G × T interactions. Genes are identified based on their house fly gene ID followed by their *Drosophila melanogaster* homologues in parenthesis. Error bars represent standard errors of the mean

T interactions affecting the expression of individual *Md-dsx* exons (Figure S13). We did not test for G × T 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; Scott et al., 2014).

We also used qRT-PCR to examine the expression of the house fly male-determining gene, *Mdmd*, in two III^M strains and two Y^M strains raised at 18°C and 27°C (Figure S14). One Y^M strain and one III^M strain originated from North America, and the other Y^M strain and III^M strain came from Europe. If temperature-dependent differential expression of *Mdmd* were responsible for the clinal distribution of Y^M and III^M (with higher expression conferring a fitness advantage), we would expect higher *Mdmd* expression in Y^M (III^M) males at lower (higher) temperatures. There was a significant G × T interaction affecting the expression of *Mdmd* in the European Y^M and III^M strains, with higher *Mdmd* expression in III^M males at lower temperatures (Figure S14). This is the opposite pattern from what would be expected if the hypothesized G × T effects on *Mdmd* expression were responsible for maintaining the cline. We observed a similar trend in the North American strains, although the interaction term was not significant. We observed these similar patterns in both population samples even though they were assayed with two different types of tissue (abdomen in the North American strains, and whole fly in the European strains), demonstrating that these results are robust to the tissues we sampled. We also did not find a significant G × T interaction affecting expression of *Md-ncm* (the ancestral paralogue of *Mdmd*), which is not part of the sex determination pathway (Figure S14). Therefore, there is no evidence that *Mdmd* expression is increased at the hypothesized favoured temperatures for Y^M and III^M males.

3.5 | G × T effects on gene expression are not driven by large-scale *cis*-regulatory divergence

We next tested if divergence of *cis*-regulatory sequences 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 were responsible for differential expression of third chromosome genes between III^M and Y^M males, the III^M and III alleles of those genes should also 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 G × T 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 were significantly differentially expressed in head or testis because of G × T interactions had concordant differences in expression between the III^M and III allele in III^M males.

To test for differences in allelic expression, we first identified 12 genes on the third chromosome with a significant G × T interaction affecting testis expression, at least one heterozygous SNP in III^M males, and homozygous at those SNP sites in Y^M males (Figure 5). 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 that we used for RNA-seq 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.

We first compared the expression of III^M and III alleles in testis. Of the 12 genes with significant G × T effects and the requisite SNPs to test for allele-specific expression, seven had a significant effect of temperature on testis gene expression within III^M males (Figure 5; Table 1). Of those seven genes, five had a pattern of allelic expression consistent with the differential expression between 18°C and 29°C within III^M males: *LOC101892094* (homologous to *D. melanogaster Pdfr*, which is responsible for regulating circadian behaviours), *LOC101891589* (homologous to *D. melanogaster CG42450*, which is predicted to be involved in G protein-coupled receptor signalling), *LOC101893483* (encoding a GATA zinc finger domain-containing protein), *LOC101891938* (homologous to *D. melanogaster mmd*, which is predicted to encode a membrane protein involved in ectodomain proteolysis), and *LOC101897352* (the cystathionine β-synthase gene associated with lifespan, mentioned earlier). Two genes had allelic expression that was inconsistent with temperature-dependent expression in III^M males: *LOC101882943* (homologous to

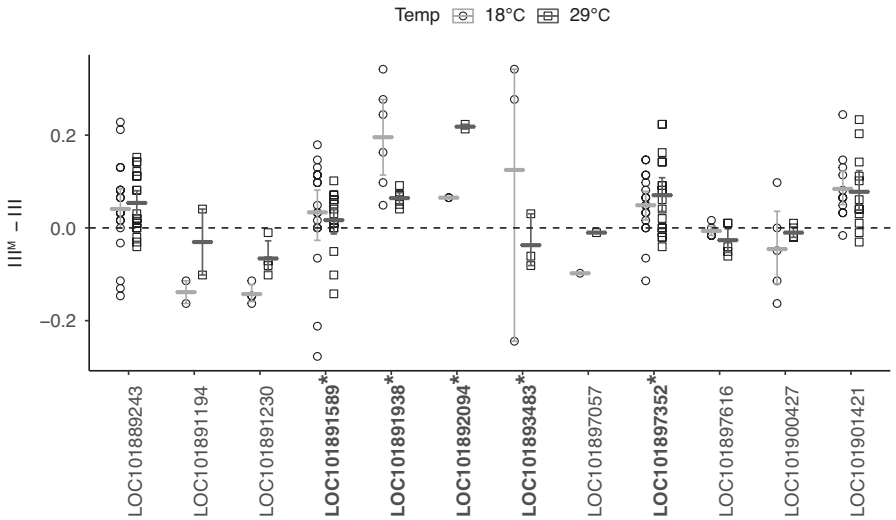

FIGURE 5 G × T interactions affect allele-specific expression in house fly testes. Differences in sequencing coverage in III^M house fly males between III^M and III alleles at either 18°C (circles) or 29°C (squares) are shown for 12 house fly genes where there is a G × T effect on testis expression between Y^M and III^M males. Each circle or square represents the difference in normalized mapped reads in testis between the III^M and III alleles at a single variable site (SNP) within a gene. Circles show expression differences between alleles at 18°C, and squares show expression differences between alleles at 29°C. The small horizontal lines indicate the mean difference in coverage between alleles across all sites in each gene at each temperature. Error bars represent the standard error across all variable sites within a gene at each temperature. The gene names with asterisks along the x-axis are differentially expressed between the III^M and III alleles in the same direction as the differential expression in III^M males between 18°C and 29°C (Table 1).

TABLE 1 Temperature-dependent allele-specific expression in testis

	Chromosome III	Rest of genome
Genes with significant temperature effect on III^M and III^M–III in right direction	5	7
Genes with significant temperature effect on III^M and incorrect direction of III^M–III	2	6
Genes with heterozygous sites in III^M males, but without a significant temperature effect on expression in III^M males	5	17

D. melanogaster Nepl5) and *LOC101900427* (homologous to *D. melanogaster fne*). The remaining five genes did not differ in testis expression between III^M males raised at 18°C and 29°C (Table 1).

To determine a null expectation for the proportion of genes with allelic expression consistent with the differential expression between 18°C and 29°C, we tested for concordance between allele-specific expression and temperature-dependent expression differences for genes on other chromosomes. We do not expect concordance for genes on other chromosomes because the inbred Y^M and III^M males used in our RNA-seq experiment share a common genetic background. We identified 30 genes on other chromosomes with heterozygous sites whose testis expression depended on the G × T interaction (Table 1). Of those 30 genes, 13 were differentially expressed between III^M males raised at 18°C and 29°C. Out of those 13 genes, seven had allele-specific expression that was consistent with the 18°C versus 29°C expression differences (Table 1). There is not a significant difference in the fraction of genes on the third chromosome whose temperature-dependent expression is consistent with changes in allele-specific expression (5/7) relative to the rest of the genome (7/13; $p = .64$ in Fisher's exact test). This suggests that the G × T effects on the 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.

When we analysed only the younger male head samples, we found seven genes on the third chromosome with a significant G × T interaction that also had at least one SNP in III^M males. Among them, only one gene (*LOC101890343*, homologous to *D. melanogaster mahe*, encoding an ATP-dependent RNA helicase) had a significant effect of temperature on gene expression within III^M males. The allele-specific expression of this gene was consistent with the temperature effect in III^M males, but there were no genes on other chromosomes with the requisite SNPs in our head RNA-seq data to test for a significant excess relative to a null expectation. When analysing all head samples, we found a single gene on the third chromosome with a significant G x T interaction that also had a SNP in III^M males. However, we did not find a significant effect of temperature on expression of this gene within III^M males.

We are limited in the analysis we can perform on allele-specific expression of genes on the X versus Y^M chromosomes because of small sample sizes. There are only 40 genes assigned to the house fly X or Y^M chromosome (Meisel & Scott, 2018), none of which had a significant G × T interaction affecting expression in testis (Table S6). Only one X or Y^M chromosome gene had a significant G × T interaction affecting expression in heads when we analysed all samples (Table S4), and it did not have any heterozygous sites. Similarly, none of the three genes on the X chromosome with a significant G × T interaction affecting expression in young male heads had any heterozygous sites.

4 | DISCUSSION

We tested how temperature affects gene expression in Y^M and III^M house fly males. These G × T effects on expression could lead to differences in temperature-dependent phenotypes between house fly genotypes. The resulting phenotypic differences could cause temperature-dependent fitness effects of the proto-Y chromosomes, which could be responsible for maintaining the Y^M-III^M latitudinal cline. Raising the flies at 18°C and 29°C exposed G × T interactions affecting gene expression in both head and testis. While these temperatures may not capture the specific conditions in which selection occurs in nature, they do allow us to assess how proto-Y genotype and temperature interact to affect phenotypes. We found no evidence that the expression levels of genes involved in the sex determination pathway were meaningfully affected by G × T interactions. In contrast, other genes throughout the genome were differentially expressed, suggesting that alleles present on either the III^M chromosome or the Y^M chromosome, other than *Mdmd*, may be targets of temperature-dependent selection.

4.1 | No evidence that G × T interactions affect the sex determination pathway in a way that explains the maintenance of polygenic sex determination

Our results suggest that G × T interactions affecting the sex determination pathway are not necessary to explain the maintenance of polygenic sex determination in house fly. We did not find evidence for G × T interactions affecting the expression of the male-determining *Mdmd* gene or exon-usage of *Md-tra* in a way that is consistent with the clinal distribution of Y^M and III^M. In addition, the expression of *Md-dsx* and *Md-fru*, the immediate downstream targets of *Md-tra*, did not depend on G × T interactions.

It is 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 (Sharma et al., 2017). Hediger et al. (2010) have shown that the *Md-tra* auto-regulatory loop can be effectively shut down in embryos by RNA interference, 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 et al., 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 differential expression of *Mdmd* that is consistent with our hypothesis for the clinal distribution of Y^M and III^M males, we believe that the increased expression of *Mdmd* in III^M males that we observe at the lower temperature 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 lower temperature 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. 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.

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

We observed some evidence that genes on the III^M chromosome, other than *Mdmd*, could affect gene expression in a way that could be responsible for temperature-dependent fitness differences between III^M and Y^M males. Notably, the third chromosome is enriched for genes differentially expressed between Y^M and III^M males (Figure 2a), consistent with previous results (Meisel et al., 2015; Son et al., 2019). This is expected as the flies differ in their third chromosome genotypes, and it suggests there are differences in *cis*-regulatory alleles between the III^M and standard third chromosomes. Consistent with this hypothesis, we observed a more pronounced clustering by genotype in our PCA when we considered only chromosome III genes (Figure S4).

The temperature-dependent effects of the III^M chromosome, however, do not appear to be mediated by large-scale *cis*-regulatory changes across the III^M chromosome for the following reasons. First, genes that were differentially expressed because of temperature were not enriched on the third chromosome in III^M males (Figure 2b). This is not because of lack of power to detect the enrichment as we saw a modest enrichment of differentially expressed third chromosome genes in young Y^M male heads (Figure S8). Third chromosome genes were also not enriched amongst those with G × T interactions affecting expression in male heads or testes (Figure S9). In addition, there was not an enrichment of third chromosome genes with temperature-dependent expression differences between the III^M and III alleles (Figure 5, Table 1). Moreover, an independent analysis of other 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, 2021). We cannot perform a similar statistical analysis of Y^M genes because of the small number of genes on that chromosome. Our results therefore suggest that widespread *cis*-regulatory differences between proto-Y and proto-X chromosomes are not responsible for G × T effects on gene expression. It is therefore more likely that a small number of loci on the proto-Y chromosomes act as temperature-dependent *trans* regulators of gene expression across the entire genome.

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

Even though a large number of *cis*-regulatory variants on the III^M chromosome cannot directly explain much of the effect of proto-Y chromosome genotype on temperature-dependent gene expression, there is evidence for temperature-dependent effects of the III^M and Y^M chromosomes which could explain their divergent phenotypic effects. First, there is some clustering by G × T combinations in the transcriptome-wide testis gene expression profiles (Figure 1c). Second, we identified substantial temperature-dependent gene expression (Figure 2b) and many genes whose expression depended on G × T interactions (Figures 3 and 4). Most of the differentially expressed genes are not on the X or third chromosomes, consistent with our hypothesis that a small number of loci on the proto-Y chromosomes act as temperature-dependent *trans* regulators of gene expression across the entire genome. 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.

Reproductive traits are a promising target of selection that could depend on G × T interactions. There were more genes differentially expressed in testis because of G × T interactions than in head, 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). Genes associated with reproductive functions (*LOC101887804*, *LOC101899763*, *LOC101894442*, and *LOC101896699*) were amongst the genes whose testis expression depended on G × T effects (Figure 4). It is therefore possible that selection along the Y^M-III^M cline acts on reproductive traits, which is consistent with the idea that the strength of sexual selection

can vary across populations (Allen et al., 2017; Arnqvist, 1992; Blanckenhorn et al., 2006; Connallon, 2015; Payne & Krakauer, 1997). These reproductive 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. Sexual antagonism is one of the few selection pressures capable of maintaining polygenic sex determination (van Doorn & Kirkpatrick, 2007; Rice, 1986). 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, 2021; Meisel et al., 2016), possibly in conjunction with epistatic interactions between either Y^M or III^M and autosomal loci not linked to either *Mdmd* locus (Schenkel, 2021). It is worth pursuing if sexual antagonism can maintain polygenic sex determination by acting on temperature-dependent gene expression differences between Y^M and III^M males.

Energy metabolism is a potential phenotype upon which selection acts to affect reproductive functions. We previously found divergence between III^M and standard third chromosome sequences surrounding genes encoding mitochondrial proteins (Son & Meisel, 2021). Here, we report G × T interactions affecting the testis expression of three genes with metabolic functions (*LOC101901027*, *LOC101901154*, and *LOC109613297*). All three genes were upregulated in Y^M males at 18°C, and, to a lesser extent, upregulated in III^M males at 29°C (Figure 4). None of the *D. melanogaster* homologues of these genes are differentially expressed between flies raised at high (21.5°C) or low (6°C) temperatures (MacMillan et al., 2016), nor are they differentially expressed between *D. melanogaster* that are evolved in hot or cold laboratory environments (Hsu et al., 2020). However, one of the metabolic genes (*LOC101901154*), encoding an aldehyde oxidase, has a *D. melanogaster* homologue (*AOX4*) that is expressed higher at 21°C than 29°C (Zhao et al., 2015), similar to the higher expression of the house fly gene in Y^M males at lower temperatures. We are cautious to interpret further because there are four tandemly arrayed *AOX* genes in the *D. melanogaster* genome and at least 3 corresponding genes in house fly; it is therefore not possible to assign orthology across this family.

One of the other metabolic genes (*LOC101901027*) encodes fructose-1,6-bisphosphatase and has a homologue (*fbp*) that is expressed higher in *D. melanogaster* raised at 29°C than those raised at 21°C, regardless of whether the flies come from Maine (USA) or Panama (Zhao et al., 2015). This is consistent with the higher expression of this gene at 29°C in III^M testes, but opposite from the lower expression at 29°C in Y^M testes (Figure 4). It is possible that the Y^M chromosome confers a fitness advantage via increased production of fructose-1,6-bisphosphatase in testes at lower temperatures. Consistent with this hypothesis, fructose-1,6-bisphosphatase is necessary for cold-stress in mice (Park et al., 2020) and associated with cold hardiness in plants and insects (Cai et al., 2018; Storey & Storey, 2012). There is also evidence that *D. melanogaster fbp* is differentially *trans*-regulated across genotypes and temperatures (Chen et al., 2015). In house fly, this gene is not found on either the Y^M or III^M chromosome, which would require it to be differentially regulated in *trans*, consistent with what is observed in *D. melanogaster*. Together with the other differentially expressed metabolic genes, our results suggest that energy metabolism related to spermatogenesis or sperm function may be a target of selection driving the evolution of the III^M and Y^M chromosomes.

Muscle performance might also be under differential selection across the Y^M-III^M cline. We identified three muscle component related genes (*LOC101893720*, *LOC101895658*, and *LOC101901052*) upregulated in Y^M male heads at 18°C (Figure 3b). One of these genes (*LOC101893720*) is homologous to *D. melanogaster bt*. Knockdown of *bt* decreases sarcomere length and reduces climbing ability in *D. melanogaster* (Perkins & Tanentzapf, 2014). Another muscle-related gene (*LOC101895658*) is homologous to *D. melanogaster Unc-89*, which encodes an obscurin protein. Reduced expression of *Unc-89* using P-element insertion results in flightless adults in *D. melanogaster* (Katzemich et al., 2012). Upregulation of these genes in Y^M males at lower temperatures might improve muscle performance.

We also found evidence that selection may act on stress tolerance across environments along the Y^M-III^M cline. A gene (*LOC101893129*) homologous to *D. melanogaster Nlaz*, which encodes an extracellular lipid binding protein (similar to apolipoprotein D and Retinol Binding Protein 4), was upregulated in heads of III^M males at the high temperature (Figure 3a). *Nlaz* is regulated by the JNK signalling pathway to confer stress and starvation tolerance, and it reduces oxidative stress by maintaining metabolic homeostasis (Hull-Thompson et al., 2009). *NLaz* mutants in *D. melanogaster* have reduced stress resistance and shorter lifespans, while overexpressing *NLaz* increases stress tolerance and extends lifespan. *Nlaz* is also upregulated at extreme low temperature in *D. melanogaster* (Chen et al., 2015; MacMillan et al., 2016). Upregulation of this gene may therefore help III^M males tolerate thermal stress at high temperatures. Consistent with this hypothesis, III^M males are more tolerant of extreme heat than Y^M males, but only if they develop at warm temperatures (Delclos et al., 2021). Our results demonstrate the utility of simultaneously studying the effects of both genotypic and temperature variation to determine how thermal stress affects gene expression (Rivera et al., 2021).

There is also evidence that improved response to thermal stress may act to increase lifespan in Y^M and III^M males at temperatures concordant with their clinal distribution. For example, *LOC101897352* encodes cystathionine β-synthase and is homologous to *D. melanogaster Cbs*. In *D. melanogaster*, *Cbs* is involved in ER stress response (Chow et al., 2013) and is a positive regulator of lifespan (Kabil et al., 2011). *LOC101897352* was upregulated in Y^M male testes at 18°C (Figure 4), consistent with longer lifespan for Y^M males at lower temperatures. This predicted temperature-dependent effect is in concordance with the natural distribution of the Y^M chromosome towards the northern (colder) end of the species' range. *LOC101897352* was also one of the genes with a consistent direction of allele-specific expression and expression difference between III^M males at 18°C and 29°C (Figure 5), providing evidence that a *cis*-regulatory allele on the III^M chromosome drives temperature-dependent expression of a gene with a potential phenotypic effect. Future work should aim to identify *cis*-regulatory

regions underlying the temperature-dependent expression differences between the III^M and III alleles in *LOC101897352* and other such genes on the third chromosome (Figure 5). Searching for such regulatory sequences in house fly is currently impeded by the lack of a chromosome-scale genome assembly and comprehensive gene annotations (Meisel & Scott, 2018; Scott et al., 2014).

Two other genes were differentially expressed in ways that are suggestive of temperature-dependent lifespan differences between Y^M and III^M males and are concordant with the latitudinal distributions of the proto-Y chromosomes. *LOC101895929* is homologous to *D. melanogaster pnt*. Knockdown of *pnt* extends lifespan in *D. melanogaster* (Dobson et al., 2019). Interestingly, we see downregulation of this gene in III^M male testes 29°C (Figure 4), suggesting longer lifespan for III^M males at a higher temperature. Lastly, *LOC101901283* is homologous to *D. melanogaster Calx*, which is associated with response to ER stress (Chow et al., 2013). *Calx* mutation reduces *D. melanogaster* lifespan (Mok et al., 2020). *LOC101901283* is upregulated in III^M male heads at 29°C (Figure 3b), suggesting a longer lifespan for III^M males at a higher temperature. All three lifespan-related genes (*LOC101897352*, *LOC101895929*, and *LOC101901283*) therefore have expression profiles consistent with longer lifespan of Y^M males at lower temperatures or III^M males at higher temperatures, suggesting that temperature-dependent senescence might be a phenotype under differential selection between III^M and Y^M males. It remains to be tested if these male genotypes have different lifespans across temperatures.

Differential expression of the aforementioned genes across proto-Y genotypes and temperatures may cause phenotypes to vary across latitudes in a genotype-dependent manner. These phenotypic differences could affect the fitness of Y^M and III^M males across the cline. Fitness differences that depend on temperature (or any geographically variable factor) can promote local adaptation and contribute to the maintenance of a stable polymorphism (Kawecki & Ebert, 2004; Levene, 1953). The infered fitness effects of the differentially expressed genes we identified is in accordance with theoretical models and other empirical data demonstrating that alleles on proto-sex chromosomes linked to the sex-determiner can drive the evolution of sex determination pathways (van Doorn & Kirkpatrick, 2007, 2010; Roberts et al., 2009). Future studies should test if the genes whose expression depends on G × T interactions do indeed affect fitness-related phenotypes.

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AUTHOR CONTRIBUTIONS

Designed research: K.A., J.H.S., D.B., L.W.B., R.P.M.; Performed research: K.A., J.H.S., A.H.R., J.J.; Analyzed data: K.A., J.H.S., A.H.R., R.P.M.; Wrote the paper: K.A., J.H.S., A.H.R., D.B., L.W.B., R.P.M.

DATA AVAILABILITY STATEMENT

RNA-seq data were collected for this manuscript and have been deposited in the NCBI Gene Expression Omnibus under accession GSE136188 (BioProject PRJNA561541, SRA accession SRP219410). Commands and additional files for analysing the RNA-seq data are available from the Texas Data Repository under https://doi.org/10.18738/T8/D14TGI.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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