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Genetic Architecture of the Thermal Tolerance Landscape in *Drosophila melanogaster*

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ABSTRACT

Increased environmental temperatures associated with global warming strongly impact natural populations of ectothermic species. Therefore, it is crucial to understand the genetic basis and evolutionary potential of heat tolerance. However, heat tolerance and its genetic components depend on the methodology, making it difficult to predict the adaptive responses to global warming. Here, we measured the knockdown time for 100 lines from the *Drosophila* Genetic Reference Panel (DGRP) at four different static temperatures, and we estimated their thermal-death-time (TDT) curves, which incorporate the magnitude and the time of exposure to thermal stress, to determine the genetic basis of the thermal tolerance landscape. Through quantitative genetic analyses, the knockdown time showed a significant heritability at different temperatures and that its genetic correlations decreased as temperatures differences increased. Significant genotype-by-sex and genotype-by-environment interactions were noted for heat tolerance. We also discovered genetic variability for the two parameters of TDT: CT_{max} and thermal sensitivity. Taking advantage of the DGRP, we performed a GWAS and identified multiple variants associated with the TDT parameters, which mapped to genes related to signalling and developmental functions. We performed functional validations for some candidate genes using RNAi, which revealed that genes such as mam, KNCQ, or robo3 affect the knockdown time at a specific temperature but are not associated with the TDT parameters. In conlusion, the thermal tolerance landscape display genetic variation and plastic responses, which may facilitate the adaptation of *Drosophila* populations to a changing world.

1 | Introduction

Environmental temperature has increased by about 1.1°C during the last century, but global warming effects have been stronger during the last 30 years (Gulev et al. 2021; Hartmann et al. 2013). This scenario is accompanied by an increase in the intensity and frequency of extreme weather events at the local scale, such as frost and heat waves (Meehl and Tebaldi 2004). These environmental changes exert important selective pressures on natural populations (Pacifici et al. 2015), leading to changes in their distribution (Lenoir et al. 2020) and potentially affecting their persistence over time (Thomas et al. 2004). These changes particularly affect ectotherms, as most already maintain a body temperature close to their thermal limit (Angilletta 2009; Deutsch et al. 2008). Given this scenario, it is important to understand the genetic architecture of thermal tolerance responsible for heritable variability therein, that is, the number of genetic variants affecting the trait, their frequencies in the population, the

magnitude of their effects, and their interactions with each other and the environment (Timpson et al. 2017). Understanding the genetic architecture of thermal tolerance will make it possible to assess the evolutionary capacity of populations to respond to global warming.

To study thermal tolerance and its genetic determinants, we need a method that can correctly determine the upper thermal limits of organisms. In this sense, there has been a controversy in recent years as to which is the most appropriate method because numerous studies have observed that the methodology used in the laboratory affects the estimates of the thermal tolerance parameters and their heritability. This makes it challenging to compare findings across studies (Castañeda et al. 2019; Chown et al. 2009; Santos et al. 2012; Sgrò et al. 2010; Terblanche et al. 2007). In general, two methods are used to determine the upper thermal limit: (1) static assays, where the organism is exposed to a constant temperature until it collapses (i.e., knockdown time), and (2) dynamic assays, where the organism is exposed to a thermal ramp until it collapses (i.e., knockdown temperature) (Beitinger and Lutterschmidt 2011). However, longer trials increase the effect of other experimental variables, such as nutrient and water availability, during the trial (Rezende et al. 2011; Santos et al. 2012). This increases the residual or environmental variability in the assays and, therefore, reduces heritability estimates (Castañeda et al. 2019). With the aim of unifying methodologies in the determination of thermal tolerance, Rezende et al. (2014) proposed using the thermal-death-time (TDT) curves to describe the thermal tolerance landscape of ectotherm species, defined as the change in survival probabilities as a function of temperature and exposure time. This integrative approach estimates the critical temperature maximum (CT_{max}) and thermal sensitivity (z) using the knockdown time obtained at different static temperatures, incorporating both the magnitude and the time of exposure to thermal stress. The thermal tolerance landscape provides a unified framework for studying thermal tolerance (Rezende et al. 2020) and links thermal tolerance to cumulative heat injury sustained under natural heat stress (Jørgensen et al. 2019, 2021; Li et al. 2023; Ørsted et al. 2022). However, little is known about the genetic determinants of the TDT curves, which limits our understanding of the evolution of the thermal tolerance landscape.

Several studies have identified genes associated with the thermal upper limits; for example, the first genes identified were those encoding heat shock proteins (HSPs), increasing their expression and reducing the detrimental effects of heat stress (Anderson et al. 2003; Dahlgaard et al. 1998; Lerman and Feder 2001; McColl and McKechnie 1999). Other genes also play a role in thermal tolerance, for example, *methuselah* (*mth*), a gene involved in longevity and stress response; *foxo*, a gene encoding for transcription factors (Araújo et al. 2013; Giannakou et al. 2004; Lin et al. 1998; Morgan and Mackay 2006); and *catsup* and *ddc*, which are genes involved in the pathway of hormones and neurotransmitters of the catecholamine family (Norry et al. 2007, 2009). The role of these genes in thermal tolerance has been validated using field release and recapture experiments (Loeschcke et al. 2011) and mutagenesis (Hoffmann and Willi 2008). In addition, many studies have described extensive lists of genes with variants associated with latitudinal or seasonal variation that may be involved in thermal tolerance (Fabian et al. 2012; Kapun et al. 2020; Kolaczkowski et al. 2011; Machado et al. 2016; Rudman et al. 2022; Zhao et al. 2015). In recent years, the development of genomic tools has facilitated the study of genetic variants associated with phenotypic traits, including the *Drosophila* Genetic Reference Panel (DGRP) (MacKay et al. 2012). The DGRP is a set of fully sequenced isogenic lines that facilitates the development of quantitative genetic analysis and genome wide association analysis (GWAS), which involves testing the association of genetic variants in the genome of multiple individuals to identify genotypic associations with a given phenotype (Tam et al. 2019). Using this genetic panel, Rolandi et al. (2018) described variants in genes related to cell organisation, cell trafficking and neurotransmitter activity, whereas Lecheta et al. (2020) classified the genes associated with thermal tolerance into three groups: (1) genes involved in protecting the organism, (2) genes involved in regulation of the thermal response and (3) genes that alter the degree of preparation of the organism's body to withstand heat stress. Although these two studies have used the DGRP to study thermal tolerance using dynamic methods, no work has been done specifically to study the genetic determinants of the parameters (CT_{max} and z) of the TDT curves.

Identifying the genes and genetic variants that affect thermal tolerance is only part of the genetic architecture; how these genetic determinants interact with the environment is critical to understanding how organisms respond to thermal variation. For instance, genotypes differ in their phenotypic responses as function of environmental conditions, which is defined as genotype-by-environment interaction (G×E) (Lazzaro et al. 2008). G×E is an important mechanism to understand the genetic variation of phenotypic plasticity (Saltz et al. 2018) and could drive the evolution of different phenotypic optima in different environments (Gillespie and Turelli 1989). Several studies have shown that thermal tolerance to low and high temperatures shows G×E (Delclos et al. 2021; Ørsted et al. 2019). In addition, there is evidence on sex differences on thermal tolerance, with females showing higher tolerance than males (Castañeda et al. 2015; Rivera-Rincón et al. 2024). However, sex differences on thermal tolerance seem to be context dependent, which could be influenced by the genetic variation (Lasne et al. 2018; Morgan and Mackay 2006). The existence of a genotype-by-sex interaction (G×S) could imply that there are selective differences between the sexes, with important implications for the evolution and maintenance of genetic diversity in natural populations, as well as the use of resources and habitats in nature (Connallon and Clark 2014; Lynch and Walsh 1998; Pennell et al. 2016; Ruzicka et al. 2020).

In this work, we used the DGRP to describe the genetic architecture of the thermal tolerance landscape in *D. melanogaster* in different thermal environments and in both sexes to understand the evolutionary potential of thermal tolerance in the face of global warming. Specifically, we used quantitative genetic analyses to estimate genetic variation, heritability, G×S, and G×E of the thermal tolerance landscape in the DGRP. Later, we identified variants and candidate genes associated with both parameters of TDT curves and finally, we performed functional validation on some of the candidate genes.

2 | Materials and Methods

2.1 | Drosophila Stocks

One hundred isogenic lines from the DGRP were used to perform the thermal assays. This subset was randomly chosen from a set of 115 DGRP available in our lab when we established the experimental design. We discard 15 lines because they have a low reproductive output to produce enough flies used in this study. The DGRP lines were generated through 20 generations of inbreeding, which were founded using females collected from Raleigh, North Carolina, United States (MacKay et al. 2012). In our laboratory, these lines were maintained at 25°C with a photoperiod of 12 L:12 D and with a food medium composed of 100 g/L of fresh yeast, 80/L g of glucose, 50 g/L of wheat flour, 11 g/L of agar, 12 mL/L of nipagin, and 6 mL/L of propionic acid. After eclosion, flies were separated by sex into new vials every 2 days. The flies used in the thermal assay were between 4 and 6 days old to control age effect on heat tolerance. Previous evidence indicates no relationship between heat tolerance and age, using flies between 9 and 22 days old (Lamb and MacDonald 1973).

2.2 | Heat Tolerance Assays

For each DGRP line, five males and five females were assayed to measure their heat knockdown time at four different static temperatures: 37°C, 38°C, 39°C, and 40°C (±0.2°C). At each temperature, flies were individually placed in a well of a modified 96-well PCR plate. This plate was placed inside a climatic chamber connected to a PELT-5 thermal controller (Sable Systems International) under soft light conditions required to video record each assay. Each trial was recorded using a web camera (Logitech), and videos were analysed using a Python script to measure each fly's movement (Pérez-Gálvez et al. 2023). Specifically, this script measures the changes in pixel density in each well and determines the frame where the last change in density occurs (i.e., the frame where the fly stops moving). This methodology allowed a rapid determination of the knockdown time of a fly exposed to thermal stress, defined here as the moment at which a fly loses the ability to perform coordinated movements. Due to the large sampling size (100 DGRP lines × 2 sexes × 4 static temperature × 5 flies = 4000 flies), this experiment was carried out in 12 runs. Briefly, seven to eight DGRP lines were assayed in each run (day), measuring the four experimental temperatures for those lines in the same day. The effect of the run (12 in total) was added on the linear mixed models to account for the variance from the experimental run.

2.3 | Quantitative Genetic Analyses of Knockdown Time

First, we analysed the effect of temperature on heat knockdown using a linear mixed model ANOVA with sex, temperature and experimental runs as fixed effects, the DGRP lines as random effects and the interaction terms. Then, we also ran reduced ANOVA models separated for each static temperature. Linear mixed models were ran using the *lme4* package for R (Bates et al. 2015). Fixed effects were tested through a type-III ANOVA and random effects were tested using a likelihood-ratio test (LRT) implemented in the *lmerTest* package for R (Kuznetsova et al. 2017). Additionally, from each reduced ANOVA model, we estimated the among-line (σ^2_L), the line × sex (σ^2_{LS}), and the within-line (σ^2_e) variance components. Using these variance components, we estimated the broad-sense heritability (H^2) and the cross-sex genetic correlation separately for each temperature as $r_{GMF}=\sigma^2_L/(\sigma^2_L+\sigma^2_{LS})$. Following Huang et al. (2020), we also estimated the amount of line × sex variance component that is due to variation among the DGRP lines in the sign and magnitude of the difference in thermal tolerance between females and males as $\sigma^2_{LS}=\sigma_{LF}\,\sigma^2_{LM}\,(1-r_{GM})+(\sigma_{LF}-\sigma_{LM})^2/2$. The first term represents the contribution of changes in the rank order of lines between sexes, and the second term represents the contribution of the sex difference in the magnitude of among-line variance.

We also estimated the broad-sense heritability and its standard error using a restricted maximum likelihood approach implemented using the MTDFREML software (Boldman et al. 1993). The broad-sense heritability (H^2) of the knockdown temperature in each static temperature was estimated as $H^2=\sigma^2_G/(\sigma^2_G+\sigma^2_E)$, where $\sigma_G{}^2$ and σ^2_E are the among-line and the within-line variance components, respectively. H^2 was sex-pooled estimated, using sex as a fixed effect. To estimate if H^2 was different from zero, we compared the maximum likelihood of the model used to estimate H^2 to the maximum likelihood from a model where the among-line variance was constrained to zero. This comparison was performed using a LRT, where the critical chi-square value was equal to 3.84 (df = 1). Genetic correlations (r_g) of knockdown time at different temperatures were estimated as $r_g=\sigma_{T1,T2}/sqrt(\sigma^2_{T1}\,\sigma^2_{T2})$, where the numerator is the genetic covariance of heat tolerance between temperatures 1 and 2, and the denominator is the square root of the genetic variance of heat tolerance at each temperature. To estimate if the genetic correlation is different from zero, we compared the maximum likelihood of the model used to estimate r_g to the maximum likelihood from a model where the genetic covariance was constrained to zero.

2.4 | TDT Curves

We calculated the average knockdown time of each static temperature, DGRP line and sex combination. These values were regressed against static temperatures following Equation (1):

$$\log_{10} t = \frac{(CT_{max} - T)}{z} \quad (1)$$

where T is the static temperature (°C), CT_{max} is the maximum critical temperature (°C), t is the collapse time (min) and z is the thermal sensitivity (Rezende et al. 2014). These curves allowed the estimation of CT_{max} by extrapolating the theoretical temperature where a collapse time of 1 min is obtained ($\log_{10} t = 0$) and the estimation of z from the slope of each TDT line ($z = -1/\text{slope}$) that describes the decay in temperature tolerance following a 10-fold increase in exposure time. We fitted a linear mixed model on CT_{max} and thermal sensitivity (z), considering sex as a fixed effect and the DGRP lines as a random effect. The interaction between DGRP lines and sex was not included in the model because our experimental design allowed the estimation of a single value of CT_{max} and z per sex and each DGRP line.

Sex effect was tested through a type-III ANOVA and DGRP line effect was tested using an LRT.

2.5 | Genome-Wide Association Analysis

We performed independent GWAS for CT_{max}, thermal sensitivity (z), knockdown time at 37°C, knockdown time at 38°C, knockdown time at 39°C, and knockdown time at 40°C to identify the variants across temperatures. All GWASs were done on sex-pooled traits. The GWASs were performed on the DGRP2 platform (MacKay et al. 2012, http://dgrp2.gnets.ncsu.edu/data.html). In summary, this analysis associates the phenotypic variation with biallelic variants present in the DGRP lines. The DGRP2 platform initially applies standard filtering to biallelic variants, selecting those with a minor allele frequency (MAF) greater than or equal to 0.05. Then, the phenotypic values are corrected by two criteria: (a) the *Wolbachia* infection status and (b) the effect of chromosomal inversion regions because homologous recombination does not occur in these regions (Huang et al. 2014). Finally, the corrected phenotypic values were analysed using ANOVAs as follows: $Y = \mu + M + L + \varepsilon$, where Y is the corrected value of the phenotype, M is the fixed effect of the marker (trait value difference between major and minor allele), L is the random effect of the DGRP lines, and ε is the residual error. Variants with a p value $< 10^{-5}$ were annotated using the FlyBase version FB5.57 (https://flybase.org). Manhattan plots were made using the R qqman package (Turner 2014).

2.6 | Gene Ontology Analysis

Gene ontology (GO) enrichment analysis was performed for candidate genes associated with CT_{max} and z. Prior to this analysis, we removed genes that do not contain any GO described in FlyBase (https://flybase.org); therefore, they are related to any functional category. The list of the filtered genes was uploaded to PANTHER version 16.0 (http://pantherdb.org), and an overrepresentation analysis was performed (PANTHER Overrepresentation Test). This analysis performs a Fisher's exact test comparing the expected frequencies in the reference genome versus the observed frequencies of each GO category. Statistical differences between expected and observed frequencies were considered only after a false discovery rate correction for multiple comparisons.

2.7 | Validation of Candidate Genes

To functionally validate the results of GWAS, we evaluated the effect of four candidate genes (*KCNQ*, *mam*, *robo3*, and *shot*) from the CT_{max} and z analysis using RNAi lines. The selection of these genes was based on the results of the gene ontology analysis, covering genes associated with the four significant categories (see 'Section 3'). These RNAi lines were obtained from the Vienna Drosophila Resource Center and Bloomington Stock Center (Table S13). We used the Tub-GAL4 driver to obtain a whole-body knockdown of each gene, with the corresponding control line for each RNAi line with the same genetic background. We measured knockdown time on each RNAi and control line following the same procedure used for the DGRP lines, using five males and five females per genotype, temperature and replicating the whole experiment three times. The knockdown time of the experiments was analysed in each sex and genotype by fitting linear mixed models using the *lmer* function from the lme4 R package (Bates et al. 2015) with the following model for each temperature: $Y = \mu + S + G + (S * G) + R + \varepsilon$, where Y corresponds to the collapse time, μ corresponds to the overall mean, S corresponds to the fixed effect of sex, G corresponds to the fixed effect of the genotype, $S * G$ corresponds to the interaction between sex and genotype, R corresponds to the random effect of the experimental replicate, and ε corresponds to the residual error.

3 | Results

3.1 | Quantitative Genetics of Knockdown Time

Full ANOVA model indicated that heat knockdown time differed significantly among temperatures ($F_{3,297} = 1275.3$, p value $< 8.55 \times 10^{-16}$; Table S1), decreased as the measurement temperature increased. The average (±SD) knockdown time was 94.7 ± 26.3 min at 37°C, 52.1 ± 16.0 min at 38°C, 30.7 ± 7.8 min at 39°C, and 21.6 ± 4.5 min at 40°C (Figure 1, Table 1). Significant variation among DGRP lines was found for knockdown time (Figure 1) at 37°C (LRT $\chi^2_1 = 57.28$, p value $= 1.39 \times 10^{-13}$), 38°C (LRT $\chi^2_1 = 85.24$, p value $< 8.55 \times 10^{-16}$), 39°C (LRT $\chi^2_1 = 45.71$, p value $= 4.45 \times 10^{-11}$) and 40°C (LRT $\chi^2_1 = 53.59$, p value $= 8.38 \times 10^{-13}$). The sex-pooled broad-sense heritability for knockdown time showed similar values across temperatures (range H^2: 0.45–0.61), and this result was independent of whether they were estimated using the mixed-linear model (Table 1) or the REML approach (Table 3). Genetic and environmental variances decreased with temperatures, but CV_L and CV_e were relatively similar across temperatures (Table 1; Table S3).

We found significant sex differences across temperatures (Figure 1, Table 1; Table S1): females tested at 37°C and 38°C showed higher tolerance than males ($F_{1,99} = 108.7$, p value $< 8.55 \times 10^{-16}$ and $F_{1,99} = 5.95$, p value $= 0.033$, respectively), whereas the males were more tolerant at 39°C and 40°C ($F_{1,99} = 24.08$, p value $= 8.58 \times 10^{-6}$ and $F_{1,99} = 51.68$, p value $= 3.58 \times 10^{-10}$, respectively). We also found high cross-sex genetic correlations at all temperatures (range r_{GME}: 0.715–0.849, Table 1), indicating a high correspondence for the heat tolerance in females and males of the same genotype. We also found a significant G × S for knockdown time at all temperatures (Figure 1, Table S1): 37°C (LRT: $\chi^2_1 = 55.96$, p value $= 2.55 \times 10^{-13}$); 38°C (LRT: $\chi^2_1 = 42.03$, p value $= 2.51 \times 10^{-10}$); 39°C (LRT: $\chi^2_1 = 60.31$, p value $= 3.45 \times 10^{-14}$) and 40°C (LRT: $\chi^2_1 = 43.07$, p value $= 1.53 \times 10^{-10}$). G×S is mainly explained by the changes in the rank order of the heat tolerance exhibited by females and males of the same DGRP line (range: 94.9%–99.96%) (Table 1), which is easily visualised by the crossing of the reaction norms for knockdown time between females and males (Figure 1).

We found significant and positive cross-temperature genetic correlations (Table 2; Table S3), which decreased as the difference between temperatures increased. The genetic correlations were greater when estimated using the REML method than when using the variance components of the mixed-linear model (Tables 2 and 3; Table S2). In addition, pairwise analysis

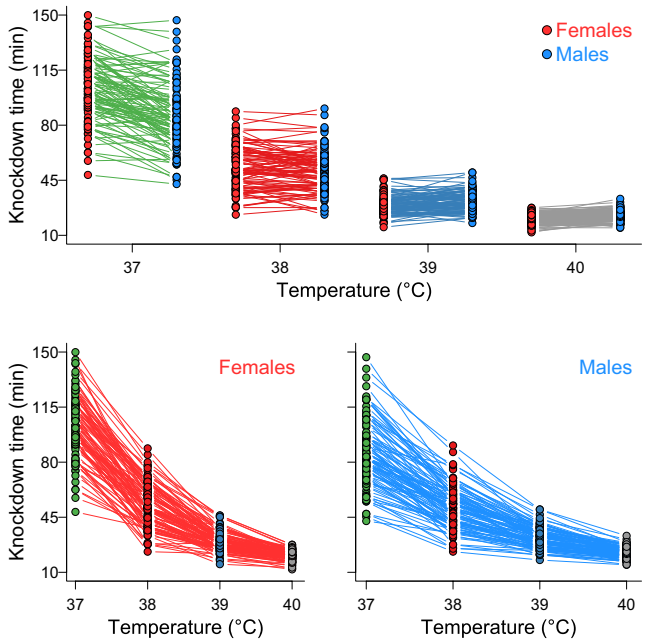

FIGURE 1 | Genotype-by-sex (top) and genotype-by-temperature in female (bottom left) and male (bottom right) reaction norms showing the effect of sex and temperature on knockdown time measured in 100 DGRP lines at different experimental temperatures. (Top) Each line corresponds to the reaction norm of a DGRP line between the mean knockdown time values in females (red circles) and males (blue circles). (Bottom) Each line corresponds to the reaction norm of a DGRP line between the mean knockdown time values of two static temperatures. [Colour figure can be viewed at wileyonlinelibrary.com]

between temperatures showed that knockdown time showed a significant genotype-by-temperature interaction (G × E), indicating genetic variation for the plasticity of knockdown time (Table S3; Figure 1). However, the source of the contribution to the G × E depended on the pairs of temperatures compared: the closer the temperatures compared, the greater the contribution of the change in the rank order of heat tolerance in G × E (Table 2). This pattern was similar for both females and males.

3.2 | Quantitative Genetics of TDT Curves

We estimated 200 TDT curves (Figure 2) with high coefficients of determination (mean $R^2 = 0.912$, range: 0.761–1.000; Table S4). CT_{max} was significantly lower for females than for males ($F_{1,99} = 142.3$, p value $< 8.8 \times 10^{-16}$; Figure 2 bottom left, Table S1): mean CT_{max} for females was 45.58°C and mean CT_{max} for males was 47.26°C. In addition, z values were significantly lower for females than for males ($F_{1,99} = 136.6$, p value $< 8.8 \times 10^{-16}$; Figure 2 bottom right, Table S1): mean z for females was 4.38°C and mean z for males was 5.45°C. The TDT curves indicated that males have higher CT_{max} (e.g., higher tolerance at acute thermal stress) than females but at the cost of exhibiting lower thermal tolerance at less extreme stress temperatures (e.g., higher tolerance at chronic thermal stress). Furthermore, both TDT parameters showed significant genetic variation among DGRP lines (Figure 2): CT_{max} (LRT: $\chi^2_1 = 39.0$, p value $= 1.14 \times 10^{-9}$) and z (LRT: $\chi^2_1 = 44.32$, p value $= 8.21 \times 10^{-11}$), and we observed crossings of reaction norms for CT_{max} and z between the sexes (Figure 2). In addition, correlations between TDT parameters were similar for females ($r = 0.96$, $t_{98} = 34.28$, p value $< 2 \times 10^{-16}$) and males ($r = 0.97$, $t_{98} = 41.74.28$, p value $< 2 \times 10^{-16}$).

3.3 | Gene and Variant Analysis

We used GWAS to identify genetic polymorphisms associated with variation in CT_{max} (Figure 3, Table 4; Figure S2), and z (Figure 3, Table 4; Figure S3). The sex-pooled GWAS for CT_{max} showed that 76 SNPs were associated within or near 61 different candidate genes (Table 4; Table S5), which were distributed throughout the entire genome with a higher concentration of SNPs located on the chromosome arms 3L and 3R, and no SNPs associated with the chromosome 4 (Figure 3). For z, the sex-pooled GWAS associated 140 variants that were highly concentrated in the chromosome arms 3L and 3R (Figure 3) and mapped within or nearby 94 candidate genes (Table 4, Table S6). For these genetic variants, 51 were shared between the two TDT parameters, which mapped to 45 candidate genes (Table 4). GO analysis (Table 6; Table S12) showed an overrepresentation of biological processes related to developmental processes and cell-to-cell signalling and an overrepresentation of genes associated with the plasma membrane and cell–cell junction (cellular component category). In addition, we found that only 5 and 16 genetic variants for CT_{max} and z, respectively, were shared between the sexes (Table 4). We also analysed the variants associated with knockdown time at the different temperatures to identify if the variants across temperatures were consistent (Table 5, Figures S4–S7, Tables S7–S10). We observed that the number of variants and candidate genes associated with the average sex-pooled knockdown time increased with temperature (Table 5). The highest number of genetic variants associated with the knockdown time was found at 40°C, mainly in chromosomes 3R and X (Figure S7, Table S10). The genetic variants and candidate genes shared between the knockdown time estimated at different temperatures were very low (Table 5), which may suggest that the genetic basis of heat tolerance is independent across temperatures. To test this, we compared the effect size of the associated SNPs between pairs of temperatures and the overlap of the 95% confidence interval (95% CI) (Table S11). In most of the cases, there was an overlap in the effect size between temperatures, with only three variants with little overlap of the 95% CI when comparing 37°C and 40°C (X_11365708_SNP: CG43155; X_18916699_SNP: CG43759; X_18916702_SNP: CG43759) and one when comparing 38°C and 40°C (3R_15226755_SNP: INO80 complex subunit or *Ino80*).

3.4 | Effect Size of Variants and Validation of Candidate Genes

We observed that most of the variants associated with CT_{max} had a negative effect size (i.e., homozygous flies for the minor allele showed higher CT_{max} than homozygous flies for the major allele). In contrast, all variants associated with z had a negative effect size (i.e., homozygous flies for the minor allele showed higher z values than flies homozygous for the major allele and, therefore, are less sensitive to change of temperature) (Figures 4 and 5). We also observed that the effect size of these variants was

TABLE 1 | Quantitative genetic and genotype-by-sex interaction parameters for knockdown time measured at four static temperatures in the *Drosophila* Genetic Reference Panel (DGRP).

	Static temperature			
Parameter	**37°C**	**38°C**	**39°C**	**40°C**
$\overline{X}$ (females)	103.15 min	53.16 min	29.36 min	20.59 min
$\overline{X}$ (males)	86.23 min	15.57 min	21.94 min	22.61 min
σ^2_L	268.38	134.56	22.20	7.45
σ^2_{SL}	82.97	23.90	8.87	2.31
σ^2_e	243.97	85.64	24.73	8.13
H^2	0.59	0.65	0.56	0.55
CV_G	26.92	31.67	25.09	19.67
CV_e	16.49	17.78	16.22	13.20
r_{GMF}	0.764	0.849	0.715	0.764
$\sigma_{LF}\sigma_{LM}(1-r_{GMF})$	82.79	24.11	8.95	2.32
$(\sigma_{LF}-\sigma_{LM})^2/2$	4.45	0.01	0.14	0.02
% rank	94.90	99.96	98.46	99.20

Abbreviations: $\overline{X}$, average value; σ^2_L, among-line variance; σ^2_{SL}, sex-by-line interaction variance; σ^2_e, within-line variance; H^2, broad-sense heritability calculated using the variance components estimated from the mixed-linear model; CV_G, coefficient of genetic variation; CV_e, coefficient of environmental variation; r_{GMF}, cross-sex genetic correlation; σ_{LF} and σ_{LM}, female and male genetic standard deviation, respectively; % rank, contribution of change in rank order of knockdown time between females and males to sex-by-line interaction variance.

inversely proportional to the frequency of the minor allele; thus, alleles that increase the thermal tolerance are at low frequency in the DGRP lines studied (Figure 4).

Regarding gene validation, we selected four genes with fly stocks readily available from the *Drosophila* Developmental Genetics Laboratory at the University of Chile. These genes represent all GO categories overrepresented among the candidate genes: developmental processes and cell-to-cell signalling (*mam*), plasma membrane (*KCNQ* and *robo3*) and cell–cell junctions (*shot*). One of these genes is candidate only for z (*KCNQ*), whereas the other three are candidates for CT_{max} and z. We found that the RNAi-*mam* line had a higher thermal tolerance than the control line at 38°C in both sexes, at 37°C only in females and at 39°C only in males (Figure 6; Table S14). On the other hand, the RNAi-*KCNQ* line showed a lower thermal tolerance than the control line at 38°C and 39°C only in males, whereas the RNAi-*robo3* line showed a lower thermal tolerance than the control lines at 40°C only in males and RNAi-*shot* lines showed higher tolerance only at 37°C in females. Finally, we found no difference between control and RNAi lines on CT_{max} and z parameters. This suggests that the candidate genes tested only affect the knockdown time at a specific temperature and in a sex-specific manner but do not affect the TDT curves.

4 | Discussion

This work reveals the genetic basis of the thermal tolerance landscape in *D. melanogaster*, which has a genetic component that can be quantified and identified through quantitative genetic and genomic analyses. We also found that heat tolerance is influenced by G × E and G × S, suggesting that the genetic architecture of heat tolerance depends on multiple factors. Our findings indicate that heat tolerance is genetically determined; however, the specific genes related to heat tolerance vary depending on temperature. The main novelty of the present study is that we combined a resource panel with high genetic variation and the thermal tolerance landscape (TDT curves) to explore the genetic architecture of thermal tolerance to high temperatures in a classic genetic and evolutionary model such as *D. melanogaster*. On the basis of significant genetic variation across temperatures, our results demonstrate that heat tolerance has enough potential to evolve under different natural selection scenarios. However, rather than genetic variation being eroded by directional selection, it can be maintained through G × E and G × S mechanisms.

4.1 | Evolutionary Potential of the Thermal Tolerance Landscape

Broad-sense heritabilities and genetic and environmental coefficients of variation for knockdown time were similar across temperatures, suggesting that this trait could undergo selection-driven evolution regardless of the thermal environment, and even though the true heritability (i.e., narrow-sense heritability) is predicted to be lower than the currently reported values (Tables 1 and 3). However, similar heritability values for thermal tolerance across temperatures were unexpected because previous studies have found that longer experiments tend to increase the effect of confounding experimental variables (e.g., starvation, desiccation) that increase the environmental variation, resulting in lower estimates of heritabilities than shorter assays (Mitchell and Hoffmann 2010;

Rezende et al. 2011). Here, the longest assay (37°C) was, on average, four to five times longer than the shortest assay (40°C), which should have affected the heritability estimates of the knockdown time. However, Rezende et al. (2011) described that the effects of desiccation and starvation are important for thermal tolerance when assays are longer than 140 min; thus, the effects of these factors should be small in our experimental design, as the longest assays lasted an average of 95 min. We also found positive genetic correlations in heat tolerance across assay temperatures, but the magnitude of the genetic correlation decreased as the difference between assay temperatures increased (Table 3, Figure S1). This pattern has been previously described for cold tolerance in *D. melanogaster* (Ørsted et al. 2019), life-history traits in *D. serrata* (Stinchcombe et al. 2010) and body size in the nine-spined stickleback (*Pungitius pungitius*) (Fraimout et al. 2022). For instance, Ørsted et al. (2019) suggested that cold tolerance has a shared genetic basis for *D. melanogaster* developed at different temperatures, but the underlying genes become more distinct as the thermal environments diverge. Here, we found differences in the effect size of the genetic variants shared between the knockdown times estimated at the different assay temperatures only when comparing the temperatures 37°C–40°C and 38°C–40°C, suggesting that the genetic basis of heat tolerance may change as the thermal environments become more different, similar to what Ørsted et al. (2019) described. This mechanism, known as environment-dependent gene action (Ørsted et al. 2019), may have significant consequences for the evolutionary predictions based on genetic information, as genes under selection could change as function of environmental conditions. Further studies involving additional genotypes and experimental replicates are needed to achieve the statistical power required to detect small differences in variant effect sizes and to thoroughly test this hypothesis. We also found a significant G×E for the knockdown time, which means that the genotypes respond differently to the thermal challenge depending on the exposure temperature. Other studies have also found significant G×E for thermal traits such as cold tolerance (Ørsted et al. 2019), and cold and heat hardiness (Fedra et al. 2019). Our findings provide valuable insights into the evolution of heat tolerance: first, genetic variation in the phenotypic plasticity of thermal tolerance should contribute to the adaptive responses if heat tolerance increases fitness in an environment-specific manner, and second, G×E should be an important mechanism to maintain the genetic diversity in natural populations if fluctuating thermal environments select different alleles contributing to heat tolerance. Thus, evaluation of fitness and genomic data in populations experiencing fluctuating environmental conditions, at spatial and/or temporal scales, is necessary to understand the adaptive role of genetic variation of phenotypic plasticity.

TABLE 2 | Genotype-by-temperature interaction parameters for knockdown time measured at four static temperatures in the *Drosophila* Genetic Reference Panel (DGRP).

Temperature comparison	Parameter			
	r_{GT}	$\sigma_{Li}\sigma_{Lj}(1-r_{GT})$	$(\sigma_{Li}-\sigma_{Lj})^2/2$	% rank
Females				
37°C, 38°C	0.586	65.34	22.73	74.73
37°C, 39°C	0.370	58.20	71.45	44.89
37°C, 40°C	0.189	42.65	101.65	29.55
38°C, 39°C	0.523	32.06	26.09	55.14
38°C, 40°C	0.337	25.32	45.39	35.81
39°C, 40°C	0.549	7.32	2.66	73.37
Males				
37°C, 38°C	0.576	109.34	28.22	79.22
37°C, 39°C	0.358	76.53	103.79	42.44
37°C, 40°C	0.213	51.63	145.33	26.21
38°C, 39°C	0.514	36.26	23.35	60.83
38°C, 40°C	0.341	27.09	44.88	37.64
39°C, 40°C	0.516	9.19	3.49	72.49

Abbreviations: r_{GT}, cross-temperature genetic correlation; σ_{Li} and σ_{Lj}, genetic standard deviation in temperatures *i* and *j*; % rank, contribution of change in rank order of knockdown time between temperatures and temperature-by-line interaction variance.

From the TDT curves, our estimates of CT_{max} were similar to those reported by Jørgensen et al. (2021) and higher than those previously described in the DGRP (Lecheta et al. 2020; Rolandi et al. 2018). These results are not unexpected because methodology has an important impact on estimates of heat tolerance (Castañeda et al. 2019; Mitchell and Hoffmann 2010). We also found that TDT parameters (CT_{max} and *z*) showed genetic variation and despite that some studies indicate that thermal tolerance has a limited evolutionary capacity (Kellermann et al. 2012 in fruit flies; Kelly et al. 2012 in crustaceans) and others support the opposite (Folk et al. 2006 in fruit flies; Geerts et al. 2015 in water fleas; see also the review by Logan and Cox 2020). In general, our results indicate genetic variation for the parameters of the TDT curves, supporting the idea that heat tolerance has the

TABLE 3 | Broad-sense heritability (diagonal; ± asymptotic standard errors) and genetic correlations (off-diagonal; ± asymptotic standard errors) between heat tolerance (knockdown time) measured at different static temperatures.

Temperature	37°C	38°C	39°C	40°C
37°C	0.47 ± 0.041			
38°C	0.82 ± 0.042	0.61 ± 0.038		
39°C	0.63 ± 0.073	0.83 ± 0.039	0.49 ± 0.041	
40°C	0.38 ± 0.098	0.62 ± 0.071	0.81 ± 0.044	0.45 ± 0.041

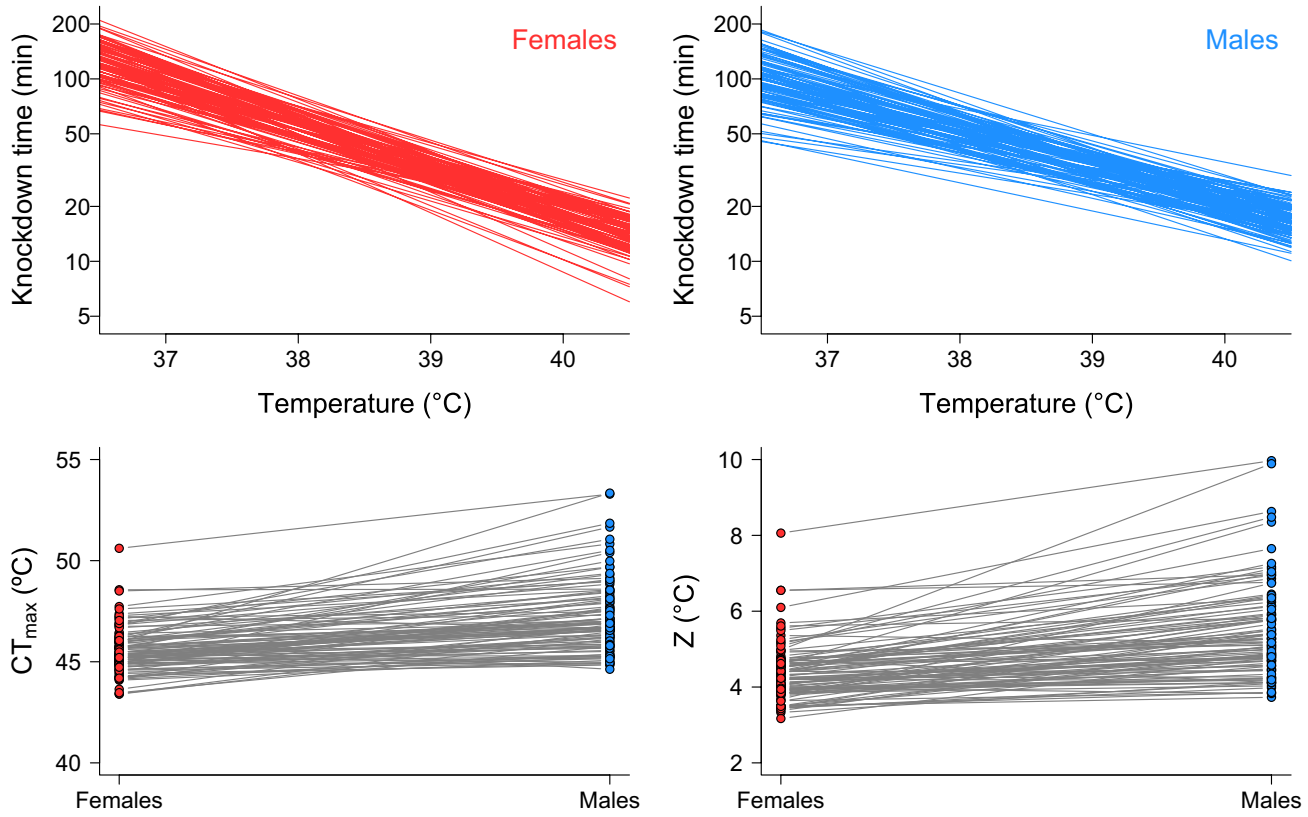

FIGURE 2 | Thermal-death-time (TDT) curves for females (upper left panel) and males (upper right panel) of 100 DGRP lines, where each line corresponds to the TDT curve of a DGRP line. Variations between sexes for the critical maximum temperature (CT_{max}) and thermal sensitivity (z) estimated from the TDT curves are shown in the lower left and lower right panels, respectively. [Colour figure can be viewed at wileyonlinelibrary.com]

potential to evolve when thermal selection acts on natural populations. This is supported by laboratory evolutionary experiments in which *Drosophila* exposed to thermal selection undergoes rapid evolutionary changes in their thermal limits (Hangartner and Hoffmann 2016; Mesas et al. 2021; Sambucetti et al. 2010).

4.2 | Genes and Variants Associated With the Thermal Tolerance Landscape

Many genetic variants associated with the parameters of the TDT curves were identified in the present study. Most of these variants are located in non-coding regions such as introns and UTR regions (see Tables S5 and S6). Genetic variation in non-coding regions has been associated with regulatory processes of gene expression and alternative splicing processes, which could affect the transcriptional plasticity and the response of populations living in changing environments (Kelly 2019; Li et al. 2021). Considering this, it is unsurprising that some of our candidate genes, such as *cac* and *fid*, exhibit differential expression between high-latitude and low-latitude populations of *Drosophila* (Zhao et al. 2015). Differential gene expression is an important mechanism exhibited by populations exposed to stressful environments, as adaptive plasticity may facilitate evolutionary rescue in these environments, 'buying time' for organisms to display a phenotypic response capable of reducing the impact of changing environments (Diamond and Martin 2021).

The genetic variants associated with the TDT curve mapped to candidate genes related to processes occurring on the periphery of the cell, such as communication between cells. For instance, we found some genes participating in synaptic processes, such as *nAChRalpha5*, which encodes a subunit of the nicotinic acetylcholine receptor (Lansdell et al. 2012); *fife*, which is associated with the neurotransmitter release (Bruckner et al. 2012); *syx6*, which is thought to be an integral component of synaptic vesicles (Gaudet et al. 2011); and *cac*, which encodes a subunit of the presynaptic voltage-gated calcium channels (Chang et al. 2014). These genetic associations suggest a relationship between the locomotor capacity of the flies and the variation observed in CT_{max} and z, which makes sense because the knockdown time is measured as the time at which a fly loses its ability to perform coordinated movements. Additionally, we also found a subset of candidate genes that are important during the fly development such as *mam* and *dl*, which participate in the Notch signalling pathway(Ahimou et al. 2004; Gomez-Lamarca et al. 2018), and *sgg*, which is involved in the canonical *Wnt* signalling pathway (Miech et al. 2008). In this category, only the *fid* gene has been implicated in the thermal resistance during the larval stage of *Drosophila* (Honjo et al. 2016). Therefore, it is possible that other candidate genes involved in larval development may affec the thermal capacity of adults. Additionally, the four candidate genes validated by RNAi showed altered knockdown times in at least one thermal condition or sex (Figure 6). Although RNAi knockdown does not recapitulate the specific

effects of the variants mapped to these genes and a direct comparison with the direction of the allele effects cannot be made, the validation indicates that changes in the expression of these genes impact the thermal phenotype of the flies.

We also found that minor alleles for most of the variants were associated with the parameters of the TDT curves, increasing the maximum critical temperature and decreasing the thermal sensitivity of *D. melanogaster* (Figure 4). This finding is easily visualised by the inverse relationship between the effect size and the frequency of the minor allele. Lecheta et al. (2020) described this pattern in the genetic polymorphisms for CT_{max} and this coincides with other phenotypes described for the DGRP (MacKay et al. 2012; Weber et al. 2012). These results could suggest at least two scenarios: (1) allele frequencies fluctuate across seasons and alleles with a large effect on CT_{max} were at low frequency when the DGRP population was established (Lecheta et al. 2020) and/or (2) alleles that have a large positive effect on thermal tolerance are interacting negatively with another fitness trait and are maintained at low frequency (Barton and Keightley 2002). On the other hand, considering that both parameters of the TDT curve are correlated and share some variants and candidate genes, it is possible that alleles contributing to high thermotolerance to acute thermal stress are under purifying selection, favoring alleles that contributing to high thermotolerance to chronic thermal stress. However, it is important to note that our analysis was conducted using a subset of the DGRP, limiting the ability to detect SNPs with small effect sizes or low allele frequencies, as a larger number of genotypes is required for such detections. Consequently, the pattern observed in Figure 4 may be a result of the limited statistical power due to the small sample size (e.g., 100 DGRP lines).

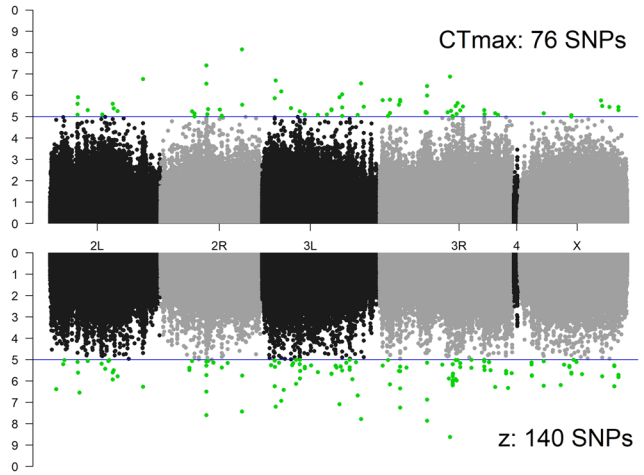

FIGURE 3 | Association between genetic variants with the mean sex-pooled values of CT_{max} (top) and z (bottom) along the *Drosophila melanogaster* genome. The blue line marks the nominal cutoff point at p value $= 10^{-5}$, and the green circles indicate the variants associated with the thermal-death-time (TDT) curves. Total number of associated variants is indicated in each panel. Details for each associated variant can be found in Tables S5 and S6 for CT_{max} and z, correspondingly. Additional GWAS on CT_{max} and z per sex can be found in Figures S2, S3 and GWAS per knockdown temperatures can be found in Figures S4–S7 and Tables S7–S10. [Colour figure can be viewed at wileyonlinelibrary.com]

Interestingly, 25% of the candidate genes reported here (27 out of 110) have been described to show latitudinal or temporal variation in previous studies of natural populations of *D. melanogaster* (Table S15). For example, four of these genes (*eip63E*, *ino80*, *MCO3*, and *egg*) showed allelic variation along a latitudinal gradient on the east coast of the United States (Fabian et al. 2012; Machado et al. 2016). Another set of candidate genes reported in the present study showed latitudinal variation in Australia (CG7720 and *Dlc90F*) (Kolaczkowski et al. 2011) and Europe (*mam*, *MCO3*, *corn* and *sug*) (Kapun et al. 2020). We also found that some candidate genes related to TDT curves showed temporal variation; for example, the genes *sgg*, *Eip74EF*, *tei*, *cac*, CG7737 and CG34354 showed positive selection between summer and autumn in a North American population (Rudman et al. 2022). Finally, several of our candidate genes showed differential expression patterns between populations from temperate and tropical climates, including genes mentioned previously such as *cac* and *fid* (Zhao et al. 2015). This suggests that these candidate genes may be under selection due to their role in the thermal tolerance to different thermal environments.

It is important to note that none of the candidate genes reported here coincide with previous ones reported in DGRP studies that used dynamic assays to estimate thermal tolerance: Lecheta et al. (2020) and Rolandi et al. (2018). However,

TABLE 4 | Results of GWAS on CT_{max} and z (TDT parameters) in the *Drosophila* Genetic Reference Panel (DGRP). Along the diagonal, each cell indicates the number of variants associated with each trait and the number of candidate genes to which they map (parenthesis). The numbers of variants and candidate genes shared between knockdown time measured at different static temperatures are indicated below and above the diagonal, respectively.

Parameter	CT_{max}	z	CT_{max} females	CT_{max} males	z females	z males
CT_{max}	76 (61)	45	15	28	18	36
z	51	140 (94)	26	22	40	49
CT_{max} females	20	40	106 (68)	8	51	8
CT_{max} males	32	24	5	59 (45)	8	31
z females	20	64	81	6	232 (145)	11
z males	45	63	10	45	16	91 (62)

both studies found genetic variants associated with developmental functions, which supports the idea that gene tuning during development could affect the capacity to withstand thermal stressors in adults. Similarly, no association with *hsp* genes was found to explain the genetic variability in the TDT curves of the DGRP lines. The proteins of the HSP family have a highly conserved sequence and structure in nature (Desai et al. 2010), and the DGRP population is likely to have low genetic variability in the genes encoding these proteins. Although these genes are important in the physiological response to thermal challenges (Feder and Hofmann 1999), genetic variation may not be at the SNP level. Indeed, *hsp70* gene expression is positively correlated with *hsp70* gene copy number, which confers a higher thermotolerance in *D. melanogaster* (Bettencourt et al. 2008).

TABLE 5 | Results of GWAS on average sex-pooled knockdown time measured at different static temperatures in the *Drosophila* Genetic Reference Panel (DGRP). Along the diagonal, each cell indicates the number of variants associated with knockdown time and the number of candidate genes they map (parenthesis). The numbers of variants and candidate genes shared between knockdown time measured at different static temperatures are indicated below and above the diagonal, respectively.

Temperature	37°C	38°C	39°C	40°C
37°C	13 (12)	3	0	0
38°C	1	11 (13)	0	1
39°C	0	0	19 (14)	2
40°C	0	0	1	37 (29)

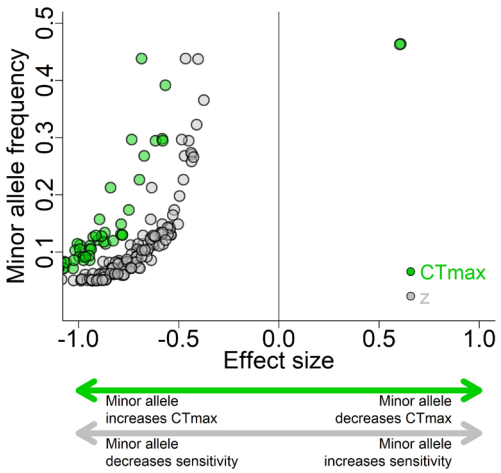

FIGURE 4 | Relationship between the minor allele frequency and effect size of variants associated with the thermal-death-time (TDT) parameters: the critical maximum temperature (CT_{max}, green circles) and the thermal sensitivity (z, grey circles). [Colour figure can be viewed at wileyonlinelibrary.com]

4.3 | Sexual Dimorphism of the Thermal Tolerance Landscape

Several works have studied the sex differences in thermal tolerance in *Drosophila*. Some authors found no significant differences between the sexes (Van Heerwaarden et al. 2016), some found higher tolerance in females (Mitchell and Hoffmann 2010) and others reported higher tolerance in males (Lecheta et al. 2020; Castañeda et al. 2015). However, all of these studies used a single static temperature to estimate the thermal tolerance. Here, we observed that the difference in thermal tolerance between sexes depends on the temperature used in the assay. If static tests are carried out at mildly stressful temperatures (≤38°C), females exhibit a higher tolerance than males. Conversely, males have a higher tolerance than females at more extreme temperatures (≥39°C). Therefore, it is important to consider the temperature used in the static assays if the study is seeking to evaluate the sexual dimorphism in thermal tolerance. Additionally, the genetic basis of the thermal tolerance landscape appears to be sex specific, given that few variants and candidate genes were shared between sexes for the parameters of CT_{max} and z.

Future studies could focus exclusively on this sexual dimorphism of the thermal tolerance landscape and even investigate the existence of genetic determinants that could have an antagonistic pleiotropic effect between sexes. The differences in the thermal tolerance landscape between the sexes could have important repercussions in natural populations. Climate change generates thermal changes associated with two different selective pressures: an increase in the global average temperature and an increase in thermal variability due to more frequent extreme events (Angilletta 2009). Considering this context, females would have a better adaptive capacity to the first scenario,

TABLE 6 | Result of gene ontology enrichment analysis of candidate genes (PANTHER Overrepresentation Test).

ID	Gene ontology	Genes	Expected	FDR
Biological processes				
GO:0008587	Imaginal disc-derived wing margin morphogenesis	5	0.32	6.79×10^{-2}
GO:0007267	Cell–cell signalling	10	1.83	6.62×10^{-2}
Cellular component				
GO:0030054	Cell junction	12	2.7	7.88×10^{-3}
GO:0005886	Plasma membrane	25	8.64	5.08×10^{-4}

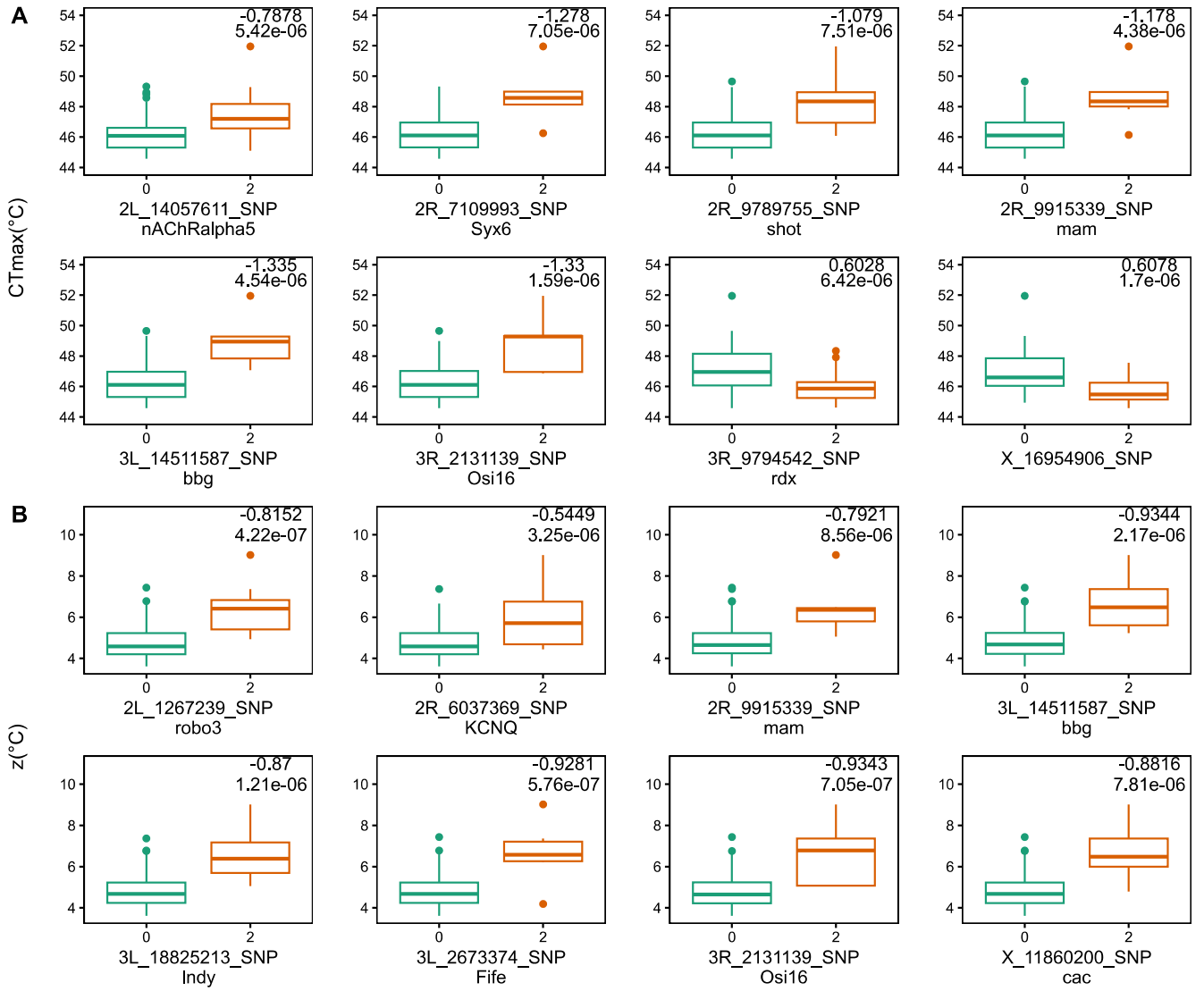

FIGURE 5 | Examples of the phenotypic effect of some variants associated with CT_{max} (A) and z (B). Variants presented were selected considering variants with high effect size (positive or negative), mapping to a candidate gene that participates in a gene ontology category significantly overrepresented (Table S12) and genes that were validated with RNAi-mediated knockdown (Figure 6). Each plot shows the phenotype distribution for the homozygous flies for the major allele (0) and the homozygous flies for the minor allele (2) for some variants with a larger effect size identified in the GWAS analyses. The x-axis shows the name of the variant and the gene it maps. Numbers in the upper right corners show the effect size (upper) and the p value of the comparison (lower). Gene names: *NAChRalpha5*, nicotinic acetylcholine receptor α5; *Syx6*, syntaxin 6; *shot*, short stop; *mam*, mastermind; *bbg*, big bang; *Osi16*, osiris 16; *rdx*, roadkill; *robo3*, roundabout 3; *KCNQ*, KCNQ potassium channel; *Indy*, I'm not dead yet; *Fife*, Fife; *cac*, cacophony. [Colour figure can be viewed at wileyonlinelibrary.com]

given their greater capacity to resist mild thermal stress (e.g., lower CT_{max} and higher thermal sensitivity). Meanwhile, males could better adapt to more variable thermal conditions, given their lower thermal sensitivity and better resistance to extreme temperatures. This idea is supported by the fact that females of *D. melanogaster* females are more resistant to mild thermal stress (Leiva et al. 2023) and prefer lower temperatures than males (Rajpurohit and Schmidt 2016). Therefore, the sexual dimorphism observed in the thermal tolerance landscape may arise from the different ways in which thermal selective pressures affect males and females.

5 | Conclusions

Studying the ability of populations to cope with environmental thermal stress through phenotypic plasticity or adaptive evolution is critical for understanding present and future shifts in species distribution and survival. In this context, the DGRP is a robust tool to study the genetic basis of the thermal tolerance landscape. Our results suggest that the genetic variation and phenotypic plasticity of the thermal tolerance should contribute to the adaptive response of *D. melanogaster* to environmental change.

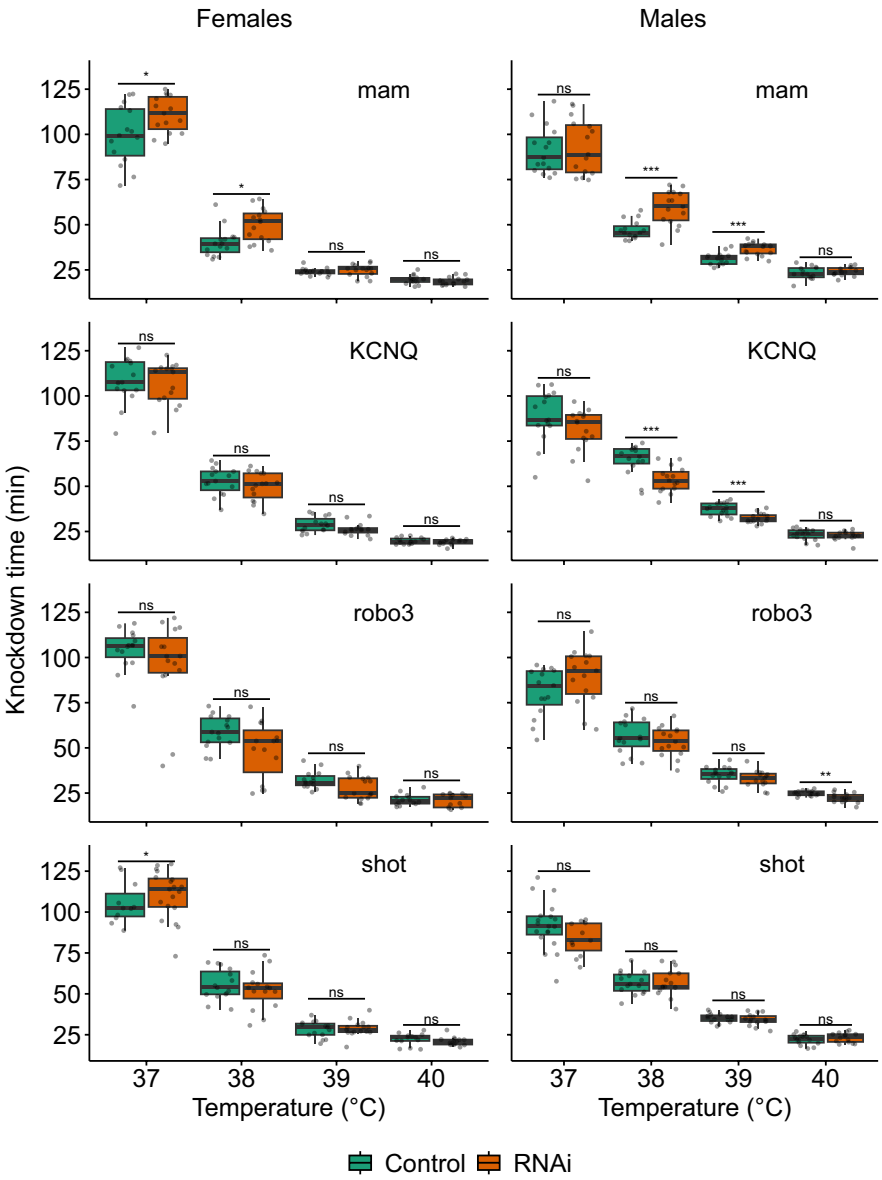

FIGURE 6 | RNAi-mediated knockdown of candidate genes associated with thermal-death-time (TDT) curves for females (left) and males (right). Symbols above boxplots represent the ANOVA results of the comparisons of the knockdown time between control (green) and RNAi (orange) lines: ns: non-significant, *p value <0.05, **p value <0.01; ***p value <0.001. The details of the statistical analysis results can be found in Table S14. The effect of these genes on CT_{max} and z in the DGRP analysis can be seen in Figure 5. [Colour figure can be viewed at wileyonlinelibrary.com]

Author Contributions

J.S. and L.E.C. conceptualised the study. J.S. performed the experiments, analysed the videos and performed statistical and genomic analyses. L.E.C. performed a quantitative genetic analysis. J.S. wrote the first draft of the manuscript, which L.E.C. reviewed and edited. P.O. facilitated the flies for the study and supervised the functional validation of candidate genes. F.P. contributed to the literature review and discussion of the study.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data are available on Figshare (https://figshare.com/s/042f264e4c8d824c6729). Codes for analyses and figures are available on Github (https://github.com/JSlox/TTL-DGRP).

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Supporting Information

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