Article Type – Investigation

Dissecting Cold Tolerance in *Drosophila ananassae*: A Multi-Phenotypic and Bulk Segregant Analysis

Vera Miyase Yılmaz[1], Fikri Tuğberk Kara[1] Sonja Grath[1*]

[1]Division of Evolutionary Biology, Ludwig-Maximilians-Universität München, Großhaderner Str. 2, 82152 Planegg-Martinsried, Germany

[*]Corresponding author: grath@bio.lmu.de

Running title: Phenotypic variation in *Drosophila*

Keywords: environmental stress, bulk segregant analysis, genetic architecture, chill coma recovery time, cold shock mortality, lethal time, thermal adaptation, QTL mapping

Abstract

As a major element of the environment, temperature impacts the geographical ranges of insects. Therefore, the worldwide distribution of insects follows adaptation to wide ranges of temperature. A population of *Drosophila ananassae* from the ancestral region showed variation in cold tolerance levels. The ancestral Bangkok population also differed from the derived Kathmandu population regarding cold tolerance. However, the phenotypic variation was only measured by chill coma assays, and no further characterization of the cold tolerance phenotype was performed. Here, we aimed to characterize further the iso-female lines of Bangkok and Kathmandu populations and the recombinant inbred lines generated from the ancestral population using lethal time and cold shock mortality in addition to chill coma recovery time. We showed that cold tolerance phenotypes differ between sexes, the additional phenotypes do not correlate significantly to chill coma recovery time, and some recombinant inbred lines have extreme phenotypes with higher tolerance than the tolerant founder or lower tolerance than the sensitive founder. We performed bulk segregant analysis using the recombinant inbred lines that exhibited extreme chill coma recovery time to identify genomic regions responsible for the phenotype. We identified 16 regions with significant association with the phenotype and showed that the genes in the putative regions were enriched in muscle development, metabolic processes, and cytoskeletal protein binding. Our results provide further evidence for the need for multiple cold tolerance phenotypes and shed light on the genetic architecture of adaptive phenotypic changes in natural populations of *Drosophila*.

Introduction

Insects can be found in all ecosystems around the globe. Surviving in diverse environmental conditions requires adaptation to various biotic and abiotic elements. As temperature is a significant and changing element of an ecosystem, the need for thermal adaptation becomes inevitable for survival in different habitats. In addition, variation in thermal tolerance also enables range expansion. Insects have various strategies to survive at temperatures outside their optimal range, which allows healthy reproduction, allowing them to tolerate or avoid freezing or, at least, tolerate chilling (Baust & Lee, 1987; Ring, 1982; Teets *et al.*, 2008, 2012; Teets & Denlinger, 2013; Wang *et al.*, 2017). However, chill-susceptible insects cannot survive extended exposure to cold (Sinclair, 1999). Investigating the genetic basis of phenotypic differences for cold tolerance is critical to understanding thermal adaptation.

Chill-susceptible insects, when exposed to cold, cannot maintain ion-water homeostasis and suffer from chilling injuries (Sinclair, 1999; Strachan *et al.*, 2011; MacMillan *et al*., 2015). When the temperature is as low as their critical thermal minimum (CTmin), they lose neuromuscular coordination and fall into a reversible chill coma. Chill coma recovery time (CCRT) can be measured once the insects are brought into warmer temperatures and used as a proxy for cold tolerance (David *et al*., 1998; Gibert *et al.*, 2001). Extended exposure to severe cold results in mortality. The mortality rate after a specific cold exposure (cold shock mortality) or the duration of exposure resulting in a 50% mortality rate (LTi50) can also be used to measure tolerance to cold (Andersen *et al.*, 2015). Even though they appear linked, the physiological mechanisms underlying these phenotypes differ (reviewed in Overgaard & MacMillan, 2017). This explains why there are multiple measures of cold tolerance and why they do not always correlate significantly.

Previous studies on insect cold tolerance have primarily focused on *Drosophila* species, which have established a worldwide distribution and undergone necessary environmental adaptations (Andersen *et al.*, 2015; Bubliy *et al.*, 2002; Colinet *et al.*, 2017; Hoffmann *et al.,* 2002; Hoffmann & Watson, 1993; MacMillan *et al.*, 2015, 2016, 2017; Parker *et al.*, 2015, 2021). Among these species, *Drosophila ananassae*, a tropical-originated species, has recently emerged as a promising model for studying the genetic architecture of cold tolerance (Königer & Grath, 2018; Königer *et al.*, 2019; Yılmaz *et al.*, 2023). This species not only expanded its geographical range but also exhibited differences in cold tolerance within and between populations (Tobari, 1993; Das *et al.*, 2004; Königer & Grath, 2018; Königer *et al.*, 2019; Yılmaz *et al.*, 2025). A population of ancestral origin from Bangkok, Thailand, showed variation among the iso-female lines, with some lines characterized as tolerant and others as sensitive based on their chill coma recovery time (CCRT). In contrast, a derived population from Kathmandu, Nepal, had less variation in CCRT, with all iso-female lines categorized as tolerant. This underscores the potential of *D. ananassae* in advancing our understanding of the genetic basis of cold tolerance.

The studies focusing on the cold tolerance of *D. ananassae* used only CCRT to characterize the cold tolerance on the ancestral Bangkok and the derived Kathmandu populations. One of these

studies performed comparative transcriptomic analysis during the recovery from chill coma, identifying genes responsible for the difference between fast and slow recovering iso-female lines from the ancestral population (Königer & Grath, 2018). Another study generated a panel of recombinant inbred lines (RILs) using the fastest and slowest recovering iso-female lines from the Bangkok population and performed a quantitative trait loci (QTL) mapping that explained 64% of the phenotypic variance (Königer *et al.*, 2019). A recent study focused on the transcriptomic response to cold acclimation that improved CCRT phenotype and identified the biochemical pathways responsible for the acclimation response in the whole body and the ionoregulatory tissues of both Bangkok and Kathmandu populations (Yılmaz *et al.*, 2025).

Bulk segregant analysis (BSA) is a cost-effective method to identify putative loci that are responsible for variation in a phenotype (Pool, 2016). By crossing two founder strains with opposite phenotypes, a recombinant population is generated exhibiting a wide range of the phenotype of interest. Instead of genotyping in large numbers, only the individuals with extreme phenotypes are sequenced and mapped as a pool (Pool, 2016; Kurlovs *et al.*, 2019). The differences in allele frequencies between the two extreme offspring pools can be used to detect putative loci (Bastide *et al.*, 2016; Bryon *et al.*, 2017; Snoeck *et al.*, 2019; Wybouw *et al.*, 2019). Benowitz *et al.* (2019) used BSA to determine the genetic architecture of *Drosophila mojavensis* locomotor activity. In another study, BSA was used to investigate the genetic basis of melanic evolution in *Drosophila melanogaster* (Bastide *et al.*, 2016). In addition to *Drosophila* species, BSA has been widely used in a variety of organisms, including other insects, yeast, and plants (Edwards & Gifford, 2012; Kurlovs *et al.*, 2019; Ma *et al.*, 2023; Magwene *et al.*, 2011; Michelmore *et al.*, 1991; Vos *et al.*, 2022).

In our current study, we have further characterized the iso-female lines from Bangkok and Kathmandu, as well as 16 RILs, using not only CCRT but also cold shock (CS) mortality and LTi50. Our findings reveal that while CCRT did not significantly correlate with CS mortality or LTi50, the latter two showed a significant correlation. Additionally, we performed a BSA mapping using the RILs with extreme CCRT, identified 16 QTL regions significantly associated with the phenotypic difference, and performed a gene ontology (GO) enrichment analysis of the putative QTLs. Finally, we suggested candidate genes responsible for the CCRT difference within the Bangkok population. Our work not only underscores the importance of using multiple measures for characterizing populations but also identifies the genetic components of cold tolerance in *Drosophila ananassae*, providing valuable insights for future research in this field.

Results

Chill Coma Recovery Time

We first measured the CCRT of flies from the Bangkok and Kathmandu populations and the recombinant inbred lines (RILs) upon 3-hour cold shock (Figure 1, Table S1). The average CCRT was 42.46 minutes for fast-recovering Bangkok lines, 61.03 minutes for slow-recovering Bangkok lines, and 55.3 minutes for Kathmandu lines. For RILs, the average CCRT was 54.62 minutes, ranging from 28 to 84.26 minutes. Two-way ANOVA suggested that sex and strain

have an effect on the phenotype as well as their interaction (Table 1). However, a paired t-test showed that the mean CCRT of the sexes was not significantly different (p-value = 0.07737) (Figure S1). The RILs with lower CCRT than the fast-founder (BKK12) or higher CCRT than the slow-founder (BKK13) lines were designated as lines with extreme phenotypes. Among the RILs, six strains showed extreme CCRT phenotypes, which were chosen for bulk segregant analysis.

Table 1: Two-way ANOVA results for CCRT phenotype using the formula that include the interaction term, *Time~Sex*Strain*. (***) indicate p-value < 0.001. The columns indicate degrees of freedom (df), sums of squares (Sum Sq), mean of squares (Mean Sq), F value, and p-value (Pr(>F)).

	df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	
Sex	1	3946	3946	13.043	0.000314	***
Strain	26	223470	8595	28.411	< 2e-16	***
Sex:Strain	26	28167	1083	3.581	3.68e-09	***
Residuals	1548	468307	303			

Mortality upon Cold Shock

Next, we focused on mortality upon cold shock. We counted the number of dead flies from the Bangkok and Kathmandu populations and the recombinant inbred lines (RILs) upon 8-hour cold shock followed by 2-hour recovery (Figure 2, Table S2). The average mortality was 28.7% for fast-recovering BKK lines, 51.3% for slow-recovering BKK lines, and 56.7% for KATH lines. For RILs, the average mortality was 39%, ranging from 10% to 86.7%. Two-way ANOVA suggested that sex and strain significantly affect the phenotype but not their interaction (Table 2). The paired t-test also showed that the mortality difference between sexes was significant (p-value = 0.02697) (Figure S2). Additionally, average mortality upon 8-hour cold shock and average CCRT showed a significant positive correlation (correlation coefficient = 0.4074743, p-value = 0.002227).

Table 2: Two-way ANOVA results for cold shock mortality phenotype using the formula that include the interaction term, *Time~Sex*Strain*. (**) indicate p-value < 0.01 and (***) indicate p-value < 0.001. The columns indicate degrees of freedom (df), sums of squares (Sum Sq), mean of squares (Mean Sq), F value, and p-value (Pr(>F)).

	df	Sum Sq	Mean Sq	F value	Pr(>F)	
Sex	1	20.8	20.765	6.908	0.00983	**
Strain	26	693.9	26.687	8.877	< 2e-16	***
Sex:Strain	26	98.2	3.778	1.257	0.20706	
Residuals	108	324.7	3.006			

Lethal Time (LTi50)

The third and last phenotype used in this study was lethal time (LTi50). Mortality rates upon various durations of cold exposure were used to calculate the duration of exposure, resulting in 50% mortality (Table S3). The average LTi50 was 10.9 hours for fast-recovering BKK lines, 8.26 hours for slow-recovering BKK lines, and 7.77 hours for KATH lines. The LTi50 of RILs ranged from 5.56 hours to 10.1 hours. Since replicates were used to calculate the LTi50 of each sex and strain, one-way ANOVA was used to test the effects of sex and strain separately. The ANOVA tests indicated that only the effect of strain on lethal time was significant (Table 3). Additionally, LTi50 showed significant correlation with average mortality upon cold shock (correlation coefficient = -0.4942833, p-value = 0. 0.0001454) but not with average CCRT (correlation coefficient = 0.03809686, p-value = 0.7845).

Table 3: One-way ANOVA results for LTi50 phenotype using the formulas, *Time~Sex* and *Time~ Strain*. (***) indicate p-value < 0.001. The columns indicate degrees of freedom (df), sums of squares (Sum Sq), mean of squares (Mean Sq), F value, and p-value (Pr(>F)).

	df	Sum Sq	Mean Sq	F value	Pr(>F)	
Sex	1	2.97	2.973	1.313	0.257	
Residuals	52	117.68	2.263			
Strain	26	104.52	4.020	6.727	2.33e-06	***
Residuals	27	16.14	0.598			

158 **Bulk Segregant Analysis**

159 Variation in cold tolerance in insects allows studying the genetic basis of cold adaptation.
160 Therefore, we performed a bulk segregant analysis on flies from recombinant inbred lines that
161 showed extreme CCRT phenotypes and flies from the founder strains. One hundred female flies
162 for each sample were pooled for whole genome sequencing. The initial number of SNPs was 1.8
163 million. These SNPs were filtered according to total depth and reference allele frequency,
164 resulting in 431 thousand SNPs. Finally, the 61 thousand SNP loci were found to be segregating
165 in all pools (Figure 3A).

166 Building on the method developed by Takagi *et al.* (2013), we calculated the allele frequency
167 differences (ΔSNP-index) within the 1 Mb windows between extremely tolerant and extremely
168 sensitive pools (Figure 3B). A total of 16 QTL regions were significantly associated with
169 phenotypic differences between the pools. Five QTL regions had negative ΔSNP-index values,
170 and 11 had positive ΔSNP-index values.

171 Using the G-prime method that is based on allele depth and takes advantage of linkage
172 disequilibrium (Magwene *et al.*, 2011), we calculated the G statistic for each SNP (Figure S3).
173 However, this analysis did not result in regions significantly associated with the phenotype.

174 **Gene Ontology Enrichment Analysis**

175 Within the significant QTL regions determined by the first analysis, 2379 genes were found. To
176 understand the roles these genes play, we performed a gene ontology (GO) enrichment analysis
177 using the *D. melanogaster* orthologs. The analysis revealed GO terms related to muscle
178 development and metabolic processes (Figure 4). When the analysis was divided according to
179 ΔSNP-index, the regions with positive values showed enrichment in muscle development,
180 metabolic processes, and cytoskeletal protein binding (Figure S4), and the region with negative
181 values showed enrichment in glucosidase, monooxygenase, and hydrolase activities (Figure S5).

182 Discussion

183 In the present study, we tested the cold tolerance of two different populations and RILs of *D.*
184 *ananassae* using CCRT, CS mortality, and LTi50. These phenotypic traits, among others, have
185 been used in many studies and shown to vary with environmental conditions (Andersen *et al.*,
186 2010, 2013, 2015; Ayrinhac *et al.*, 2004; Colinet *et al.*, 2013; David *et al.*, 1998, 2003; Hallas *et*
187 *al.*, 2002; Hoffmann *et al.*, 2002; Hoffmann and Weeks, 2007; Kobey and Montooth, 2013;
188 Nilson *et al.*, 2006; Sisodia and Singh, 2010, 2012). A previous study by Andersen *et al.* (2015)
189 tested the cold tolerance of 14 *Drosophila* species using five measures to identify which of these
190 measures correlated with the environmental variables. Previous analysis suggested that four iso-
191 female lines of the Bangkok (BKK) population were slow-recovering with high CCRT, four iso-
192 female lines of the same population were fast-recovering with low CCRT, and three iso-female
193 lines of Kathmandu (KATH) population were fast-recovering with low CCRT (Königer & Grath,
194 2018). Our tests on the iso-female lines indicated that the average CCRT of the fast-recovering
195 BKK lines was lower than both slow-recovering BKK and KATH lines. However, further

196 characterization revealed that the males of the slow-recovering BKK lines were more tolerant
197 (lower CS mortality and higher LTi50) than the males of the KATH lines, whereas females were
198 less tolerant. High variation in these phenotypic traits between sexes and among iso-female lines
199 in each group was also observed. This approach indicated the importance of using multiple
200 phenotypes when characterizing iso-female lines, populations, and species regarding complex
201 traits.

202 Recombinant inbred lines showed continua of CCRT, LTi50, and CS mortality. For CCRT, some
203 RILs showed extreme phenotypes on both ends. In other words, some RILs recovered from chill
204 coma faster than the fast-founder BKK12 strain, and some recovered slower than the slow-
205 founder BKK13 strain. On the other hand, the RILs showed extreme LTi50 and CS mortality
206 only on the sensitive end, exceeding the values of BKK13. However, it should be noted that
207 CCRT was used to determine the founder strains, and LTi50 and CS mortality of the iso-female
208 lines were not measured prior to the generation of the RILs. The extreme phenotypes and the
209 continuum of phenotypic traits suggest that cold tolerance is a polygenic trait with more than one
210 dominant causal allele.

211 The chill coma recovery assay also revealed that the CCRT of RILs was inconsistent with the
212 previous measurement performed by Königer *et al.* (2019). The line numbers given according to
213 the initial CCRT values did not align with the average CCRT measured in the current study. The
214 changing phenotype can result from high genetic variation within and among the lines and
215 changes in allele frequencies due to bottleneck effect or inbreeding. Therefore, understanding the
216 genetic architecture of CCRT phenotype in the RILs becomes more prominent. The bulk
217 segregant analysis requires pools of segregating populations – RILs in the current study – with
218 contrasting phenotypes to determine the genetic basis of the trait (Ehrenreich *et al.*, 2010). These
219 populations are expected to be dissimilar in the putative regions but similar in regions unlinked
220 to the trait (Kurlovs *et al.*, 2019; Pool, 2016). The RILs that exhibit extreme phenotypes allow
221 such analysis in the current study. Therefore, we selected the individuals from RILs with
222 extremely low or extremely high CCRT.

223 We attempted to identify the genomic regions associated with the phenotypic difference between
224 extremely cold tolerant and extremely cold sensitive flies using two methods: Δ(SNP-index) and
225 G' analyses. The association plots indicate that Δ(SNP-index) and G' value peaks align in
226 various regions. However, the analyses showed discrepant results: the former analysis revealed
227 16 QTL regions associated with the phenotypic difference between the two bulks, whereas the
228 latter analysis did not show any significant regions after multiple testing correction. As these two
229 analyses use different calculations and statistical methods, the sensitivities of the two analyses
230 are different: Δ(SNP-index) can detect SNPs with high allele frequency difference even when the
231 region is isolated, whereas G' analysis requires consistency across the region (Magwene *et al.*,
232 2011; Takagi *et al.*, 2013). Further validation of the QTL regions detected by Δ(SNP-index)
233 analysis but not by G' analysis is necessary. The SNPs in one of these regions exhibited p-values
234 lower than 0.05 prior to multiple testing correction, making the region candidate for further
235 validation. This 682-kb region on chromosome 2R contained genes enriched in muscle

development, chitin binding, and palmitoylation (Figure S6, Table S4). Among the 19 chitin-binding genes, *D. melanogaster* orthologs of *GF25314* and *GF24516* (*CG17147* and *Muc68D*, respectively) were previously associated with cold acclimation (MacMillan *et al.*, 2016), *D. melanogaster* orthologs of *GF24478* and *GF24481* (*CG6933* and *CG7298*, respectively) had high or very high expression in response to cold, but not associated with cold tolerance before. In contrast, the remaining 15 genes were not associated with cold. A previous study suggested chitin binding as a significant player in the acclimation response of *D. ananassae* (Yılmaz *et al.*, 2025). The expression of chitin-binding genes increased in both cold-tolerant and sensitive strains in response to cold acclimation, with a significant difference between the phenotypes. In the current study, the GO analysis results not only align with the previous suggestion but also provide a genetic explanation of the differential expression between the phenotypes. Two genes that contribute to muscle development are *polo* and *rt*. While *polo* was previously associated with hypoxia tolerance (Azad *et al.*, 2012; Gilliland *et al.*, 2009), the human ortholog of *rt* was shown to be responsible for muscle dystrophy (Jurado *et al.*, 1999). The four *D. melanogaster* genes associated with palmitoylation in the GO analysis were orthologs of a single *D. ananassae* gene, *GF24551*.

Gene ontology analysis of the *D. melanogaster* orthologs of the genes within the 16 QTL regions revealed similar biological processes and molecular functions, such as muscle development and palmitoylation. Chill coma recovery is a complex process that includes recovery of the membrane potential and restoration of the ion-water balance, eventually resulting in regain of neuromuscular coordination (reviewed in Overgaard & MacMillan, 2017). As the gain of muscle control is essential for chill coma recovery, variation in the genes involved in muscle development may lead to differences in CCRT. Thirty-four genes, including 20 *His2A* orthologs, were involved in muscle development and were within the significant QTL regions associated with the phenotypic difference. Among these genes, *Obscurin* and *Msp300* were also related to cytoskeleton protein binding. Previous studies suggested the cytoskeleton as an essential element of response to cold stress (Königer & Grath, 2018; Königer *et al.*, 2019; Yılmaz *et al.*, 2025; Cottam *et al*., 2006; Kim *et al*., 2006; von Heckel *et al*., 2016; Chen *et al*., 2017; Des Marteaux *et al*., 2018; Bowman *et al*., 2018). Genes related to actin cytoskeleton, for instance, showed differential expression in response to cold shock (Königer & Grath, 2018) and cold acclimation (Yılmaz *et al.*, 2025). The cytoskeleton impacts the cell structure, shape, and migration and acts as an anchor to ion channels (Denker & Barber, 2002). Studies have shown that microtubules and actin polymers affect the calcium concentration in the cell. *Obscurin* encodes a titin-like protein responsible for the symmetrical assembly of the sarcomere (Schnorrer *et al.*, 2010). *Msp300* encodes a protein responsible for positioning the muscle nuclei, mitochondria, and neuromuscular junction (Volk, 1992). Neither of these two genes was previously associated with cold tolerance phenotypes. On the other hand, *klarsicht*, a gene related to cytoskeleton protein binding, was previously suggested as a candidate gene that may play a role in the phenotypic difference of fast- and slow-recovering BKK strains (Königer *et al.*, 2019). Like *Msp300*, the protein product of *klarsicht* is responsible for positioning the nuclei in muscles. Two more genes were within the QTL regions significantly associated with the phenotypic difference between the

bulks, *GF14829* (*D. melanogaster*/*CG10383*) and *GF21827* (*D. melanogaster*/*Arpc3B*). These two genes were not found in the GO analysis; however, the former is involved in adult longevity and sleep (Donggi *et al.*, 2012), and the latter is involved in actin binding (Hudson & Cooley, 2002). Both genes were previously associated with phenotypic differences within the BKK population (Königer & Grath, 2018; Königer *et al.*, 2019).

Palmitoylation is the attachment of a palmitic acid – a 16-carbon fatty acid – to cysteine residues of a protein by thioester bonds (Linder & Deschenes, 2007). Its roles include regulation of protein localization, protein-protein interactions, signal transduction, and protein stability. Palmitoylation also affects membrane fluidity by regulating protein localization in the plasma membrane. Due to these characteristics and functions, palmitoylation becomes an essential element of cold tolerance. Nine *D. ananassae* genes found within the significant QTLs showed enrichment in protein palmitoylation. Previous studies showed that four genes (Table S4) were expressed in the central nervous system (Bannan *et al.*, 2008).

Exposure to low temperatures causes protein denaturation, energy deficiency, and decreased membrane fluidity (Overgaard & MacMillan, 2017; Teets & Denlinger, 2013). Extended exposure to cold leads to chill injury and, eventually, insect death. Previous studies on chill susceptible insects suggest that maintaining ion homeostasis is key to cold tolerance (Andersen *et al.*, 2017; Overgaard *et al.*, 2021). Disturbance in the ion-water balance causes loss of membrane potential, which leads to impairment of muscle excitability (Overgaard & MacMillan, 2017). However, it is uncertain if the loss of neuromuscular coordination results from failure in neurons, muscles, or synapses. Our study suggests that in addition to ion transport, fatty acid metabolism, protein folding, and cellular respiration, cold tolerance of chill susceptible insects also depends on muscle development and palmitoylation in the central nervous system and provides further evidence for the roles of the cytoskeleton and chitin-binding in chill coma recovery at the genetic level.

Given the established genome editing technology within the species by Yılmaz *et al.*, (2023), we suggest 16 candidate genes (Table S4) that can be edited and used in validation assays, including phenotype evaluation, electrophysiological techniques, and reabsorption measurements. Undoubtedly, functional studies can underpin the roles of candidate genes in chill coma recovery and cold tolerance. In addition, selection experiments using the fly strains with contrasting genotypes can explain the selection processes acting on the genetic variation. Overall, by integrating different phenotypic measurements and genome-wide variant analysis, our study brings the underlying mechanisms of chill coma recovery into light and enhances our understanding of cold tolerance in the model system *Drosophila*.

Material and Methods

Fly Husbandry

This study used eight *Drosophila ananassae* iso-female lines from Thailand (Bangkok, BKK), three iso-female lines from Nepal (Kathmandu, KATH), and sixteen recombinant inbred lines generated previously by Königer *et al.* (2019) using two Bangkok lines as founders. All iso-

316 female lines were collected in 2002 (Das *et al*., 2004). The fly strains were maintained under
317 standard laboratory conditions (Rearing temperature – RT: 22˚C ± 1˚C 14:10 hours light:dark
318 cycle) at low density on corn-molasses medium. All experiments were performed on flies that
319 were expanded in large vials for two generations under the same conditions.

320 **Cold Tolerance Phenotype Measurements**

321 For all phenotype measurements, the flies of the F2 generation were collected in 0-to-2 days
322 upon emergence. After a day of recuperation, the flies were anesthetized under brief exposure to
323 CO2, separated according to sex and placed into small vials with fresh fly food in groups of 10
324 individuals. The flies were allowed to recuperate another day after sex separation and tested
325 when 4-6 days old. Three replicate vials are used per sex and strain for all phenotyping assays.

326 *Chill Coma Recovery Time*

327 The 4-6-day old flies were transferred into empty vials without CO_2 exposure and placed on
328 melting ice at 0˚C for 3 hours to induce chill coma. The flies were placed into rearing
329 temperature and the time of recovery -regain of the ability to stand up- was recorded for 90
330 minutes. The flies that recovered after 90 minutes were recorded as 92 minutes and the dead flies
331 were discarded from the assay.

332 *Cold Shock Mortality & LTi50*

333 The 4-6-day old flies were transferred into empty vials without CO2 exposure and placed on
334 melting ice at 0˚C for different time periods ranging between 1-24 hours spanning 0-100%
335 mortality. At the end of cold exposure, the flies were placed into rearing temperature for 2 hours
336 to allow recovery from chill coma. The number of dead flies in each vial was recorded. The
337 number of dead flies upon 8-hour exposure to melting ice was taken into consideration for cold
338 shock mortality. For LTi50, the time of exposure corresponding 50% mortality of each sex and
339 strain was calculated from overall mortality data.

340 *Data Analysis*

341 LTi50 was calculated using generalized linear models and quasibinomial distribution in R
342 version 4.2.2 (R Core Team, 2022). The effects of line and sex on cold tolerance phenotype was
343 tested using two-way ANOVA as implemented in R.

344 **Sample Collection and DNA Extraction**

345 Four pools were generated to be used in bulk segregant analysis. For parental pools, 100 females
346 were collected from F2 generations of each founder. For recombinant inbred line pools with
347 extreme phenotypes, the flies from the F2 generation of selected RILs were collected in 0-to-2
348 days upon emergence. After a day of recuperation, the flies were anesthetized under brief
349 exposure to CO2 and separated according to sex. Single female flies were placed into small vials
350 containing fresh fly food. The 4-6-day old flies were placed on melting ice at 0˚C for 3 hours to
351 induce chill coma. Then, the vials were returned to the rearing temperature for recovery and
352 checked every 30 minutes. The flies that could stand in the first 30 minutes were collected as

353 extremely fast-recovering individuals. The flies that could stand in the second 30 minutes were
354 discarded. At the end of the third and the last 30 minutes, the living flies were collected as
355 extremely slow-recovering individuals regardless of their recovery state. The chill coma recovery
356 assay was repeated until 100 flies were collected for each extreme pool. The flies were placed
357 into 1.5mL Eppendorf tubes in groups of 10 and snap-frozen in liquid nitrogen for DNA
358 extraction. The DNA was extracted using MasterPure™ DNA Purification Kit (Epicentre,
359 Madison, WI, USA).

360 **Whole Genome Sequencing**

361 The DNA samples were pooled together and sent for whole genome sequencing to an external
362 facility (Novogene, Cambridge, UK), which performed high-throughput sequencing using a
363 Novaseq 6000 sequencer (Illumina, San Diego, CA, USA) to generate 150-bp paired-end reads.
364 On average 62.4 million reads were generated per sample. The raw data was mapped to
365 *Drosophila ananassae* genome (NCBI *Drosophila ananassae* Annotation Release 102) using the
366 Burrows-Wheeler Aligner tool (version 0.5.0). Further processing -sorting, merging, duplicate
367 removing- was done using samtools (version 1.18). Variant calling was performed using
368 freebayes (version 1.3.8). The variants were filtered using bcftools (version 1.18). Finally,
369 GATK-VariantsToTable tool was used to prepare the data for further analysis on R (version
370 4.2.2).

371 **Bulk Segregant Analysis**

372 The analysis was limited to both arms of chromosomes 2, 3, and X, discarding unlocalized
373 scaffolds. Using the QTLseqr package (version 0.7.5.2) (Mansfeld and Grumet 2018) in R, a
374 QTL analysis was performed, calculating number of SNPs in 1 Mb windows and Δ(SNP-index),
375 which corresponds to the alternate allele frequency difference of high and low bulk. Using the
376 same package, G statistics of each SNP as well as the G' values in 1 Mb windows were
377 calculated. The high and low bulks were chosen as extremely tolerant RIL pool and extremely
378 sensitive RIL pool, respectively. The QTL regions that showed significant association with
379 phenotypic differences between the bulks were annotated using the annotation file provided with
380 the reference genome.

381 **Gene Ontology Enrichment Analysis**

382 The genes within the significant regions were identified. To determine the GO terms associated
383 with the *D. melanogaster* orthologs of these genes, GO enrichment analysis was performed using
384 clusterProfiler (version 4.6.2) (Wu *et al.*, 2021) implemented in R. The p-values were corrected
385 using FDR method with a p-value cut-off of 0.01. The significant GO terms were visualized
386 using the genekitr package (version 1.2.5) (Liu, 2023).

387 Data Availability

388 Whole genome sequencing data are available at NCBI SRA archive under the accession number
389 PRJNA1247020. All other data and scripts are available in the article and in its Supplementary
390 Material.

Author Contributions

Conceptualization S.G.; sample collection V.M.Y., F.T.K.; investigation V.M.Y., F.T.K.; writing-original draft V.M.Y.; analysis and visualization V.M.Y.; funding acquisition S.G.

Acknowledgements

We thank Hilde Lainer and Bianca Hölldobler for technical assistance in the laboratory, Amanda Glaser-Schmitt and John Parsch for their valuable input on experimental design, and Dirk Metzler for the help regarding statistical analyses. We extend our appreciation to all current and previous members of the Division of Evolutionary Biology, as well as the Graduate School Life Science Munich (LSM) at LMU Munich.

Funding

Our work was supported by the Deutsche Forschungsgemeinschaft (project 271330745 to S. G.).

Conflicts of Interest

The authors declare no conflicting interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

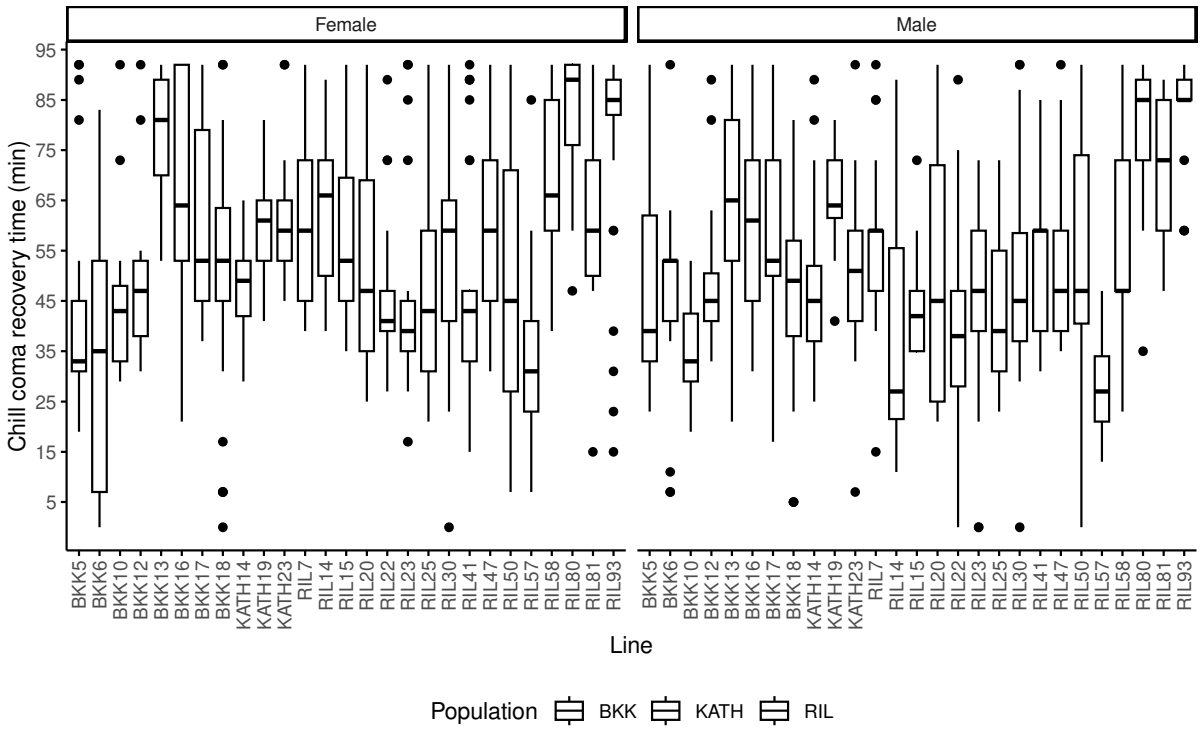

Figure 1: Chill coma recovery time (in minutes) of iso-female and recombinant inbred lines in female (left panel) and male (right panel) flies. The colors indicate the origin of the iso-female lines: Bangkok (purple), Kathmandu (blue), recombinant inbred lines (green). The black dots indicate outliers. Thirty flies were tested per sex and strain in groups of ten.

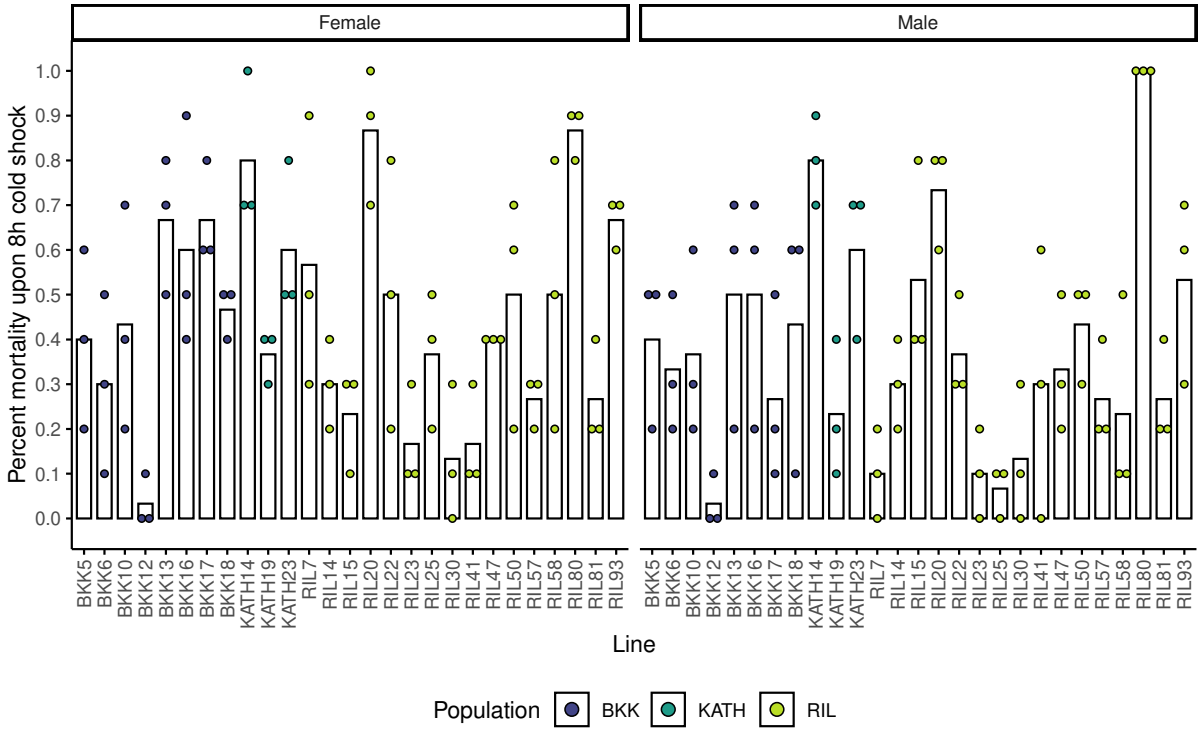

Figure 2: Percent mortality of iso-female and recombinant inbred lines in female (left panel) and male (right panel) flies measured after 8h cold shock followed by 2h recovery. The colors indicate the origin of the iso-female lines: Bangkok (purple), Kathmandu (blue), recombinant inbred lines (green). The dots indicate mortality rate in three replicates.

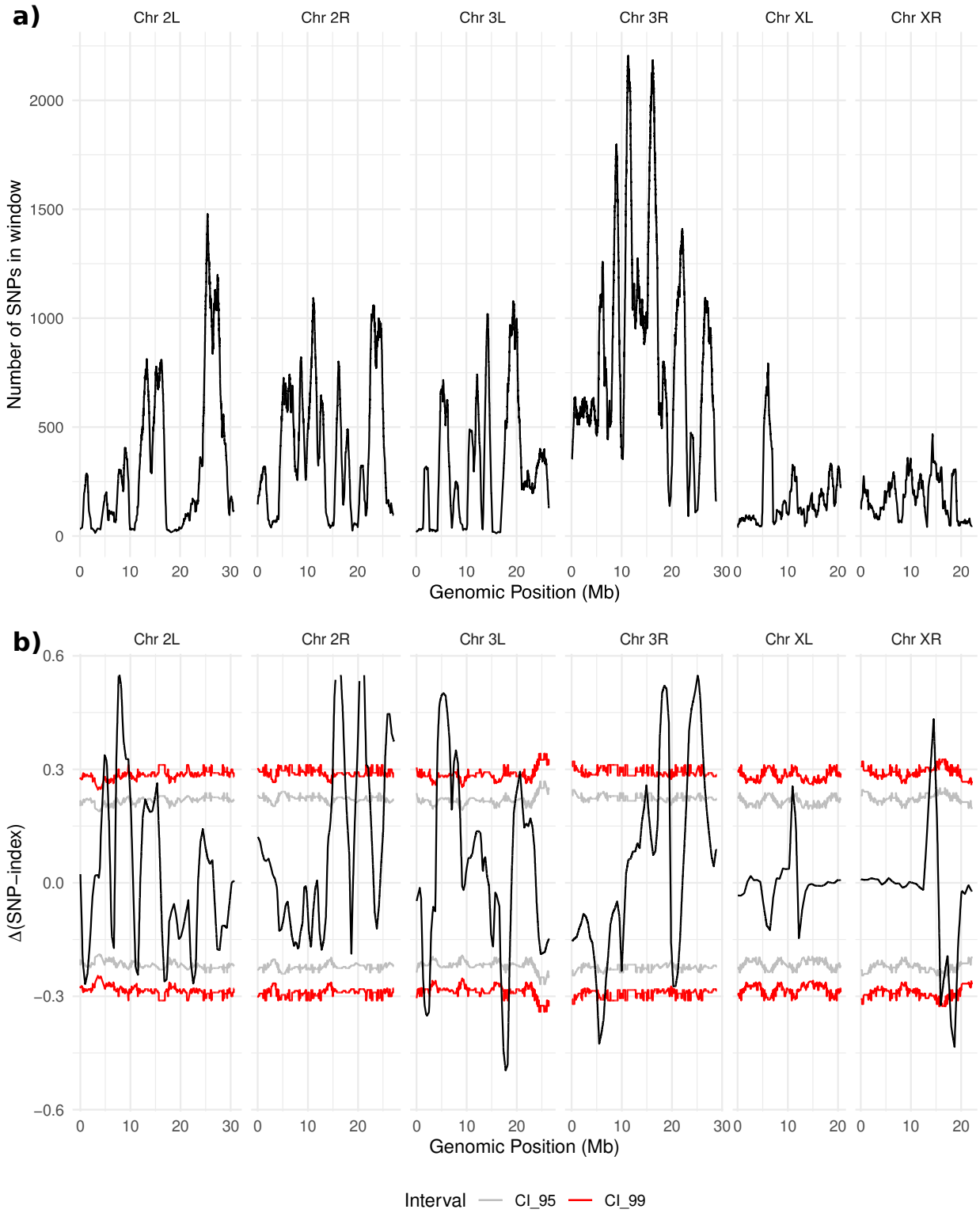

Figure 3: QTL-Seq analysis. **A)** SNP density across genome within 1 Mb windows. **B)** ΔSNP-index plot across the genome. Confidence intervals (CI) shown in grey (95%) and red (99%). Significant QTLs were determined using 99% CI.

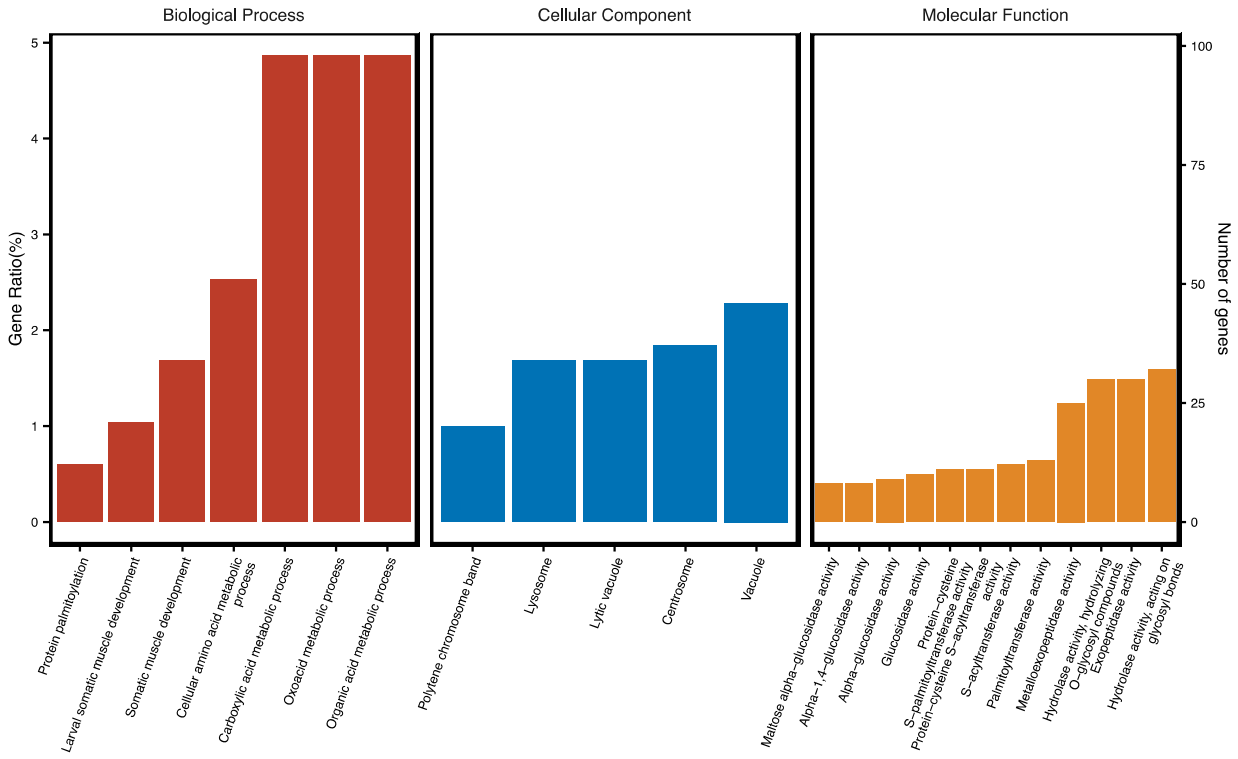

Figure 4: Gene ontology enrichment analysis for genes within the significant QTL regions. Biological processes shown in red, cellular components shown in blue and molecular functions shown in yellow.

Supplementary Tables

Table S1: Mean chill coma recovery time (CCRT) and standard deviation of female and male flies of strains. RIL-founder strains are shown in red.

	Females		Males	
Line	**Mean CCRT (min)**	**St Dev**	**Mean CCRT (min)**	**St Dev**
BKK5	41.68966	21.118887	47.03333	18.659348
BKK6	32.40000	25.685901	46.48276	17.072578
BKK10	43.36667	13.329985	34.20000	8.331080
BKK12	47.36667	13.110889	47.13333	12.681057
BKK13	77.63333	13.327398	66.20000	16.922542
BKK16	66.06667	23.094907	61.41379	18.298160
BKK17	61.33333	19.740267	60.06667	19.351438
BKK18	49.56667	24.012473	45.93333	19.692346
KATH14	47.46667	8.923944	46.73333	14.342926
KATH19	60.93333	10.136766	65.66667	8.856454
KATH23	60.33333	11.117967	50.70000	15.170639
RIL7	61.03333	19.336375	54.50000	15.440096
RIL14	64.40000	15.004827	36.60000	20.249478
RIL15	57.20000	17.856516	43.73333	9.677323
RIL20	51.50000	19.530259	48.50000	23.836729
RIL22	45.86667	13.793385	39.30000	19.818400
RIL23	44.20000	17.983517	46.13333	17.930389
RIL25	49.06667	20.282374	42.31034	15.196577
RIL30	53.36667	21.504584	49.36667	20.472830
RIL41	48.16667	22.087730	51.41379	14.266638
RIL47	59.96667	17.901037	51.25000	14.940933
RIL50	48.83333	24.280094	52.35714	26.087054
RIL57	35.00000	15.058507	28.00000	8.878490
RIL58	68.10714	18.174747	59.33333	18.775136
RIL80	82.56667	12.350299	81.21429	11.939220
RIL81	61.10000	15.114163	73.13333	12.724490
RIL93	76.10714	22.236672	84.25926	9.928950

Paired *t*-test comparing mean CCRT in males and female: p-value = 0.07737
(t = -1.839, df = 26)

Table S2: Mean mortality upon 8-hour cold shock and standard deviation of female and male flies of strains. The mortality represents number of dead flies in groups of 10. RIL-founder strains are shown in red.

	Females		Males	
Line	**Mean Mortality**	**St Dev**	**Mean Mortality**	**St Dev**
BKK5	4.00	2.0000000	4.00	1.7320508
BKK6	3.00	2.0000000	3.33	1.5275252
BKK10	4.33	2.5166115	3.67	2.0816660
BKK12	0.33	0.5773503	0.33	0.5773503
BKK13	6.67	1.5275252	5.00	2.6457513
BKK16	6.00	2.6457513	5.00	2.6457513
BKK17	6.67	1.1547005	2.67	2.0816660
BKK18	4.67	0.5773503	4.33	2.8867513
KATH14	8.00	1.7320508	8.00	1.0000000
KATH19	3.67	0.5773503	2.33	1.5275252
KATH23	6.00	1.7320508	6.00	1.7320508
RIL7	5.67	3.0550505	1.00	1.0000000
RIL14	3.00	1.0000000	3.00	1.0000000
RIL15	2.33	1.1547005	5.33	2.3094011
RIL20	8.67	1.5275252	7.33	1.1547005
RIL22	5.00	3.0000000	3.67	1.1547005
RIL23	1.67	1.1547005	1.00	1.0000000
RIL25	3.67	1.5275252	0.67	0.5773503
RIL30	1.33	1.5275252	1.33	1.5275252
RIL41	1.67	1.1547005	3.00	3.0000000
RIL47	4.00	0.0000000	3.33	1.5275252
RIL50	5.00	2.6457513	4.33	1.1547005
RIL57	2.67	0.5773503	2.67	1.1547005
RIL58	5.00	3.0000000	2.33	2.3094011
RIL80	8.67	0.5773503	10.00	0.0000000
RIL81	2.67	1.1547005	2.67	1.1547005
RIL93	6.67	0.5773503	5.33	2.0816660

Paired *t*-test comparing mean mortality in males and female: p-value = 0.02697
(t = -2.3444, df = 26)

Table S3: Lethal time (LTi50) -in hours- for female and male flies of BKK, KATH, and RIL strains. RIL-founder strains are shown in red.

Line	Female	Male
BKK5	8.62	9.62
BKK6	8.99	12.63
BKK10	13.16	11.79
BKK12	11.46	11.11
BKK13	8.16	10.12
BKK16	7.04	7.98
BKK17	7.62	9.17
BKK18	7.48	8.50
KATH14	6.09	6.38
KATH19	9.28	9.81
KATH23	7.46	7.61
RIL7	8.02	8.99
RIL14	8.51	7.98
RIL15	8.62	7.75
RIL20	6.94	7.15
RIL22	8.42	8.61
RIL23	8.86	9.33
RIL25	8.93	9.66
RIL30	9.21	8.99
RIL41	9.38	8.92
RIL47	8.17	8.4
RIL50	7.75	7.1
RIL57	9.23	10.1
RIL58	8.05	8.88
RIL80	6.09	5.56
RIL81	8.98	9.95
RIL93	7.41	8.51

637 **Table S4:** Candidate gene list.

Candiadate gene	*D. melanogaster* ortholog	GO term	Cold tolerance association
GF12043	*Obsc*	Muscle development Cytoskeletal protein binding	-
GF14378	*Msp300*	Muscle development Cytoskeletal protein binding	-
GF24896	*klar*	Cytoskeletal protein binding	Königer *et al.*, 2019
GF14829	*CG10383*	-	Königer *et al.*, 2019
GF21827	*Arpc3B*	-	Königer & Grath, 2018
GF12009	*CG1407*	Protein palmitoylation	
GF13019	*CG10344*	Protein palmitoylation	
GF14793	*CG18810*	Protein palmitoylation	
GF17953	*CG5196*	Protein palmitoylation	
GF24551	*CG4956, CG17198, CG17196, CG17195*	Protein palmitoylation	-
GF25314	*CG17147*	Chitin binding	MacMillan *et al.*, 2016
GF24516	*Muc68D*	Chitin binding	MacMillan *et al.*, 2016
GF24478	*CG6933*	Chitin binding	-
GF24481	*CG7298*	Chitin binding	-
GF10050	*polo*	Muscle development	-
GF24536	*rt*	Muscle development	-

638 **Note:** The GO analysis was performed using the *D. melanogaster* orthologs of the *D. ananassae* genes (column:
639 Candidate genes).

Supplementary Figures

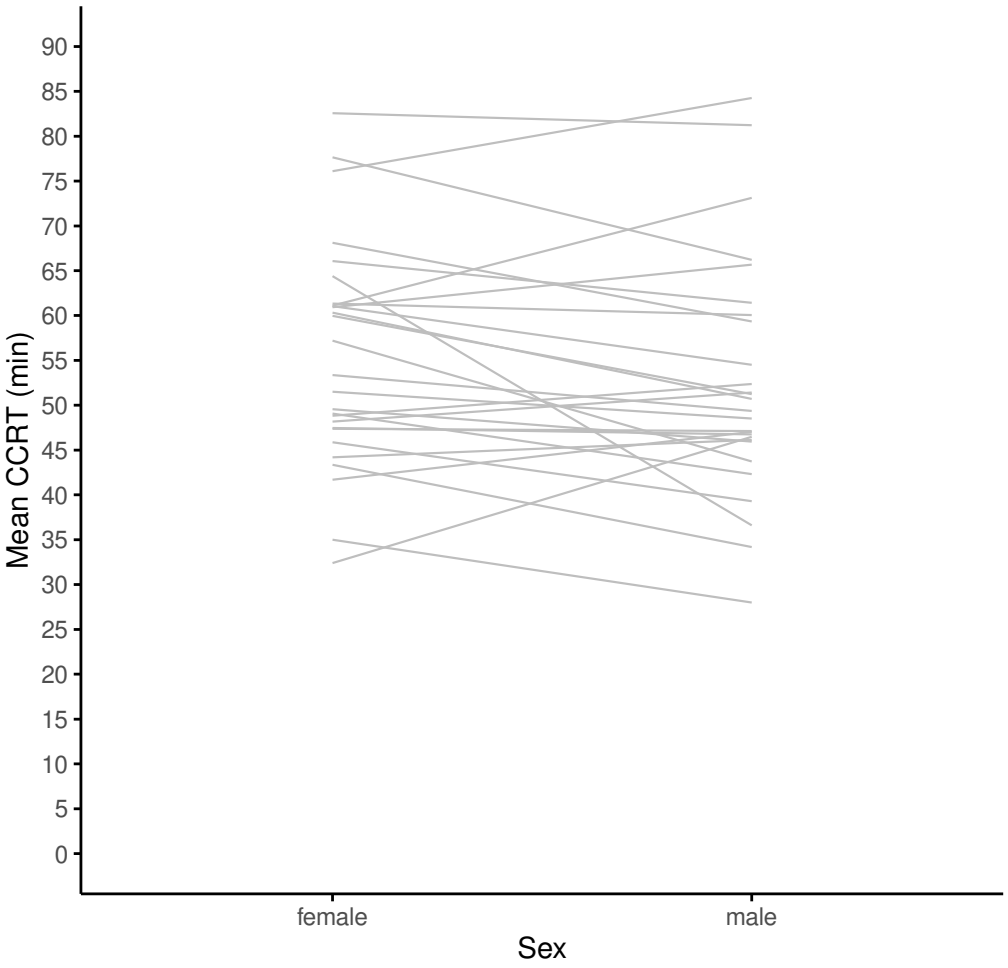

Figure S1: Boxplot for mean CCRT. Males (orange) and females (purple) of the strains are connected with grey lines.

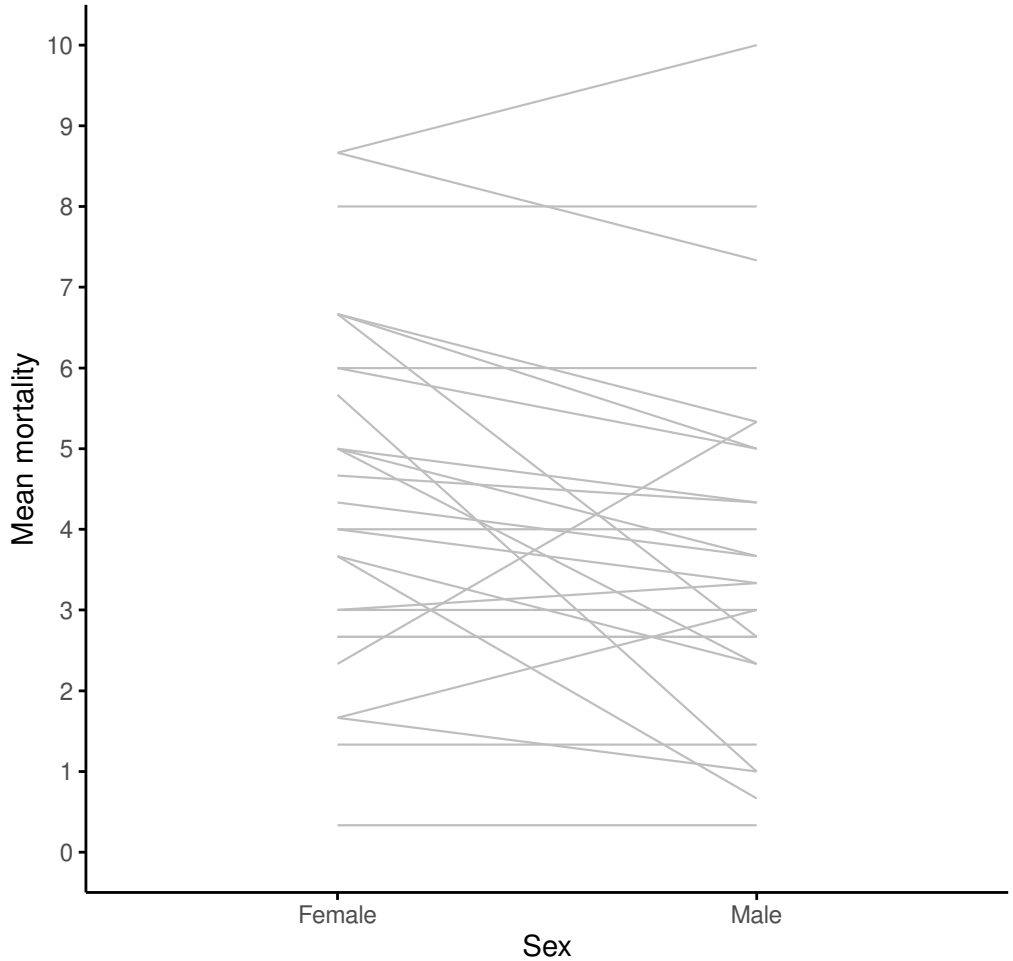

Figure S2: Boxplot for mean mortality upon 8-hour cold shock. Males (orange) and females (purple) of the strains are connected with grey lines.

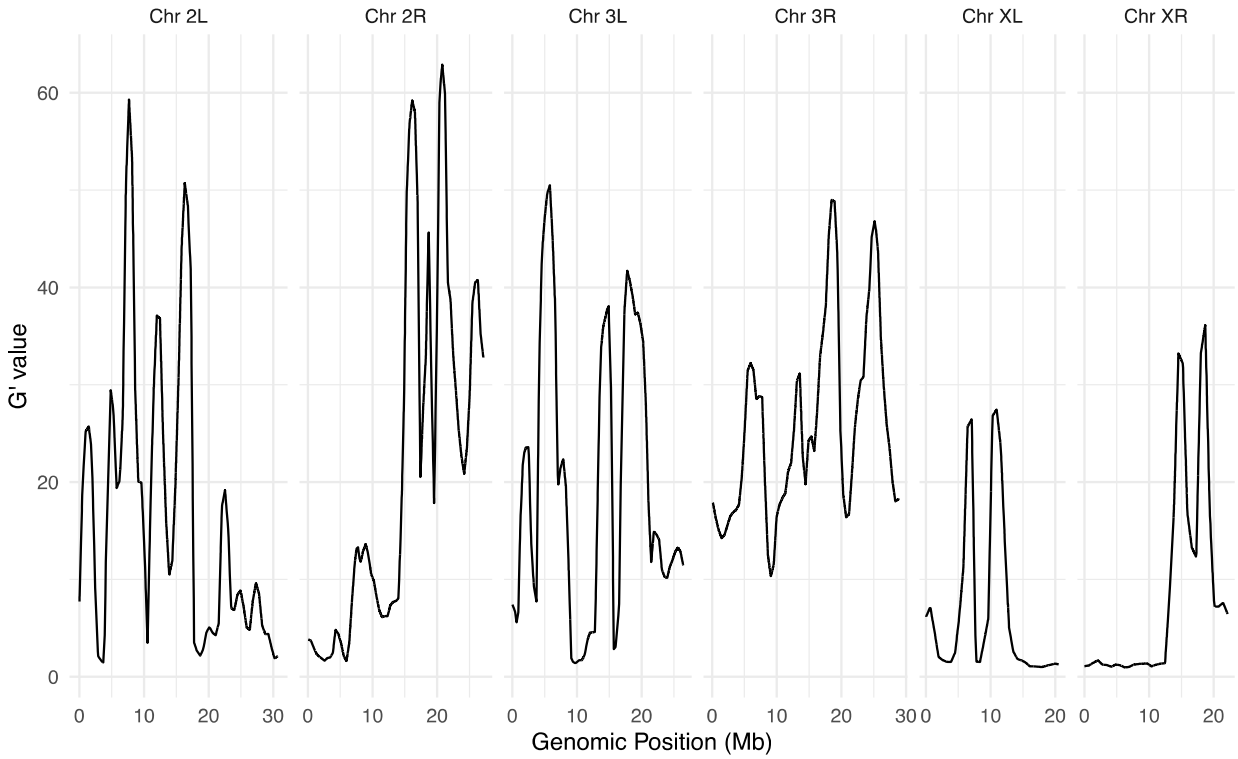

Figure S3: Association plot for each chromosome arm: sliding window (1 Mb) G' analysis. x-axis indicates genomic position on each chromosome arm, y-axis indicates G' value. FDR = 0.01 (not shown).

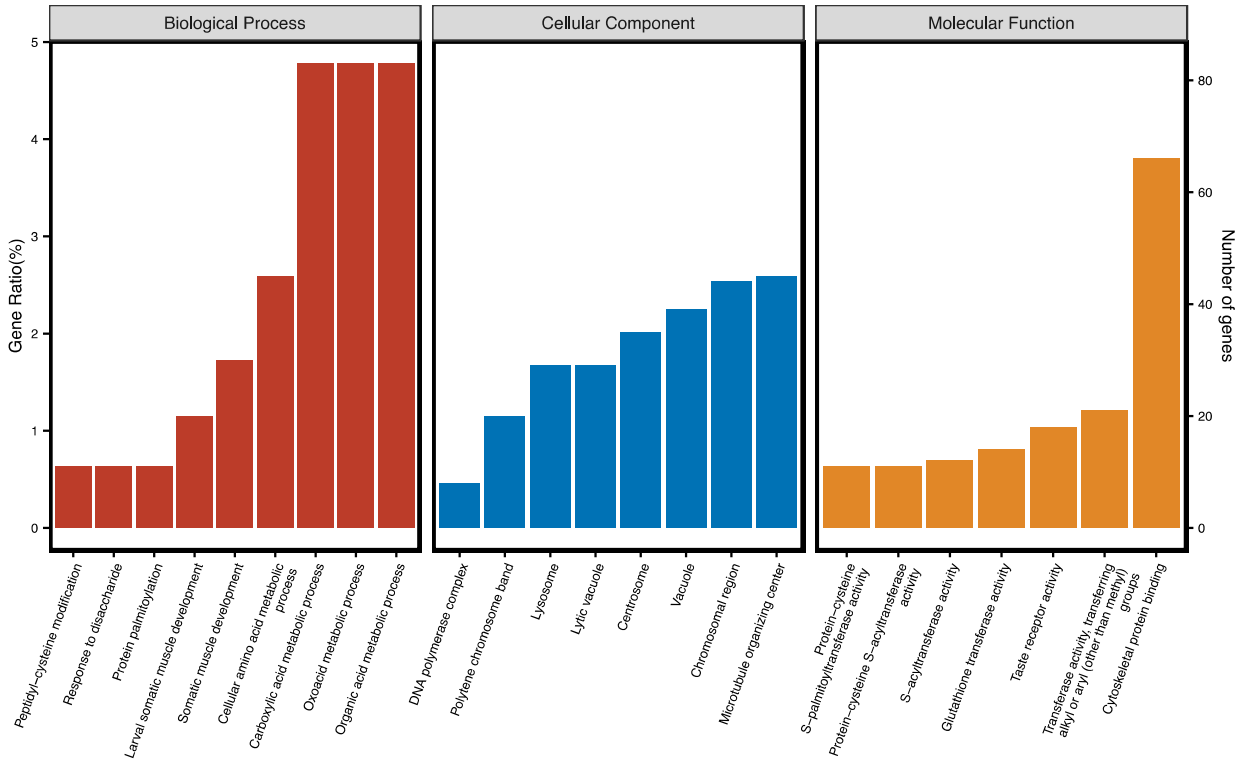

Figure S4: Gene ontology enrichment analysis for genes within the significant QTL regions that had positive ΔSNP-index values. Biological processes shown in red, cellular components shown in blue and molecular functions shown in yellow.

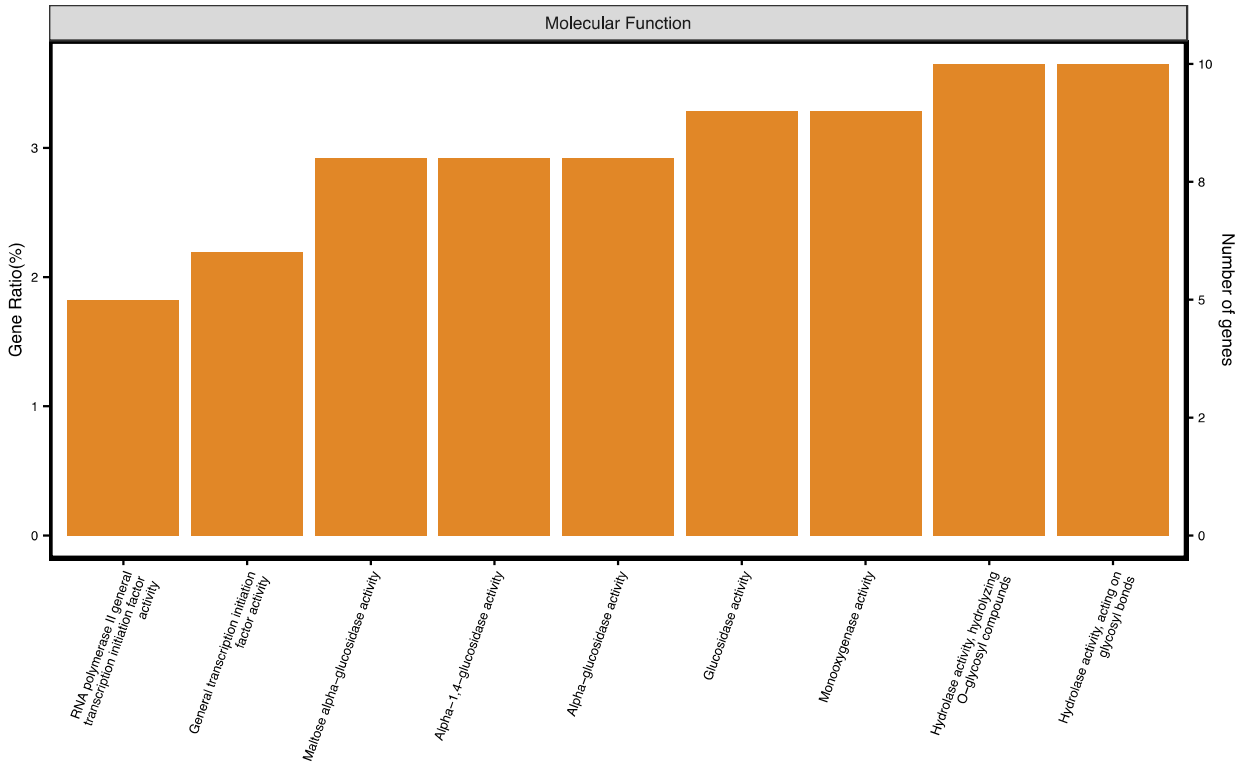

Figure S5: Gene ontology enrichment analysis for genes within the significant QTL regions that had negative ΔSNP-index values. Molecular functions shown in yellow.

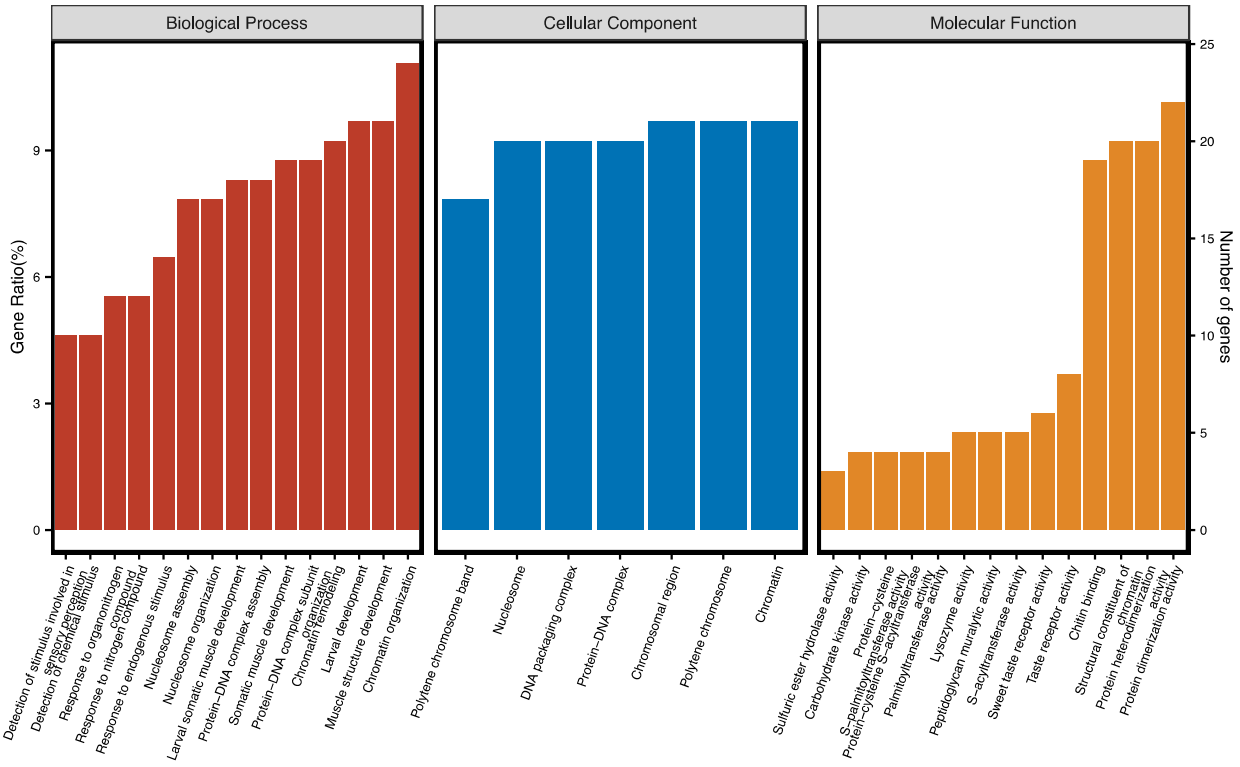

Figure S6: Gene ontology enrichment analysis for genes within the 682-kb region shared by the Δ(SNP-index) and G' analyses. Biological processes shown in red, cellular components shown in blue and molecular functions shown in yellow.