Article Type – Investigation

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

Vera Miyase Yılmaz[1], Vincent Ohlhauser[1], Fikri Tuğberk Kara[1] and 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 further characterize 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 them to adapt 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). Thererfore, investigating the genetic basis of phenotypic differences for cold tolerance is critical for 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).

Here, we have further phenotypically characterized cold tolerance for the iso-female lines from Bangkok and Kathmandu, as well as for 16 RILs, determining 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 negative 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	3,946	3,946	13.043	0.000314	***
Strain	26	223,470	8,595	28.411	< 2e-16	***
Sex:Strain	26	28,167	1,083	3.581	3.68e-09	***
Residuals	1548	468,307	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 = -0.494, p-value = 0.000145) but not with average CCRT (correlation coefficient = 0.0381, p-value = 0.785).

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			

Bulk Segregant Analysis

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

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

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

Gene Ontology Enrichment Analysis

Within the significant QTL regions determined by the first analysis, 2,379 genes were found. To understand the roles these genes play, we performed a gene ontology (GO) enrichment analysis using the *D. melanogaster* orthologs. The analysis revealed GO terms related to muscle development and metabolic processes (Figure 4). When the analysis was divided according to ΔSNP-index, the regions with positive values showed enrichment in muscle development, metabolic processes, and cytoskeletal protein

binding (Figure S4), and the region with negative values showed enrichment in glucosidase, monooxygenase, and hydrolase activities (Figure S5).

Discussion

In the present study, we tested the cold tolerance of two different populations and RILs of *D. ananassae* using CCRT, CS mortality, and LTi50. These phenotypic traits, among others, have been used in many studies and shown to vary with environmental conditions (Andersen *et al.*, 2010, 2013, 2015; Ayrinhac *et al.*, 2004; Colinet *et al.*, 2013; David *et al.*, 1998, 2003; Hallas *et al.*, 2002; Hoffmann *et al.*, 2002; Hoffmann and Weeks, 2007; Kobey and Montooth, 2013; Nilson *et al.*, 2006; Sisodia and Singh, 2010, 2012). A previous study by Andersen *et al.* (2015) tested the cold tolerance of 14 *Drosophila* species using five measures to identify which of these measures correlated with the environmental variables. Previous analysis suggested that four iso-female lines of the Bangkok (BKK) population were slow-recovering with high CCRT, four iso-female lines of the same population were fast-recovering with low CCRT, and three iso-female lines of Kathmandu (KATH) population were fast-recovering with low CCRT (Königer & Grath, 2018). Our tests on the iso-female lines indicated that the average CCRT of the fast-recovering BKK lines was lower than both slow-recovering BKK and KATH lines. However, further characterization revealed that the males of the slow-recovering BKK lines were more tolerant (lower CS mortality and higher LTi50) than the males of the KATH lines, whereas females were less tolerant. High variation in these phenotypic traits between sexes and among iso-female lines in each group was also observed. This approach indicated the importance of using multiple phenotypes when characterizing iso-female lines, populations, and species regarding complex traits.

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

The chill coma recovery assay also revealed that the CCRT of RILs was inconsistent with the previous measurement performed by Königer *et al.* (2019). The line numbers given according to the initial CCRT values did not align with the average CCRT measured in the current study. The changing phenotype can result from high genetic variation within and among the lines and changes in allele frequencies due to bottleneck

effect or inbreeding. Therefore, understanding the genetic architecture of CCRT phenotype in the RILs becomes more prominent. The bulk segregant analysis requires pools of segregating populations – RILs in the current study – with contrasting phenotypes to determine the genetic basis of the trait (Ehrenreich *et al.*, 2010). These populations are expected to be dissimilar in the putative regions but similar in regions unlinked to the trait (Kurlovs *et al.*, 2019; Pool, 2016). The RILs that exhibit extreme phenotypes allow such analysis in the current study. Therefore, we selected the individuals from RILs with extremely low or extremely high CCRT.

We attempted to identify the genomic regions associated with the phenotypic difference between extremely cold tolerant and extremely cold sensitive flies using two methods: Δ(SNP-index) and G' analyses. The association plots indicate that Δ(SNP-index) and G' value peaks align in various regions. However, the analyses showed discrepant results: the former analysis revealed 16 QTL regions associated with the phenotypic difference between the two bulks, whereas the latter analysis did not show any significant regions after multiple testing correction. As these two analyses use different calculations and statistical methods, the sensitivities of the two analyses are different: Δ(SNP-index) can detect SNPs with high allele frequency difference even when the region is isolated, whereas G' analysis requires consistency across the region (Magwene *et al.*, 2011; Takagi *et al.*, 2013). Further validation of the QTL regions detected by Δ(SNP-index) analysis but not by G' analysis is necessary. The SNPs in one of these regions exhibited p-values lower than 0.05 prior to multiple testing correction, making the region candidate for further 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-female lines were collected in 2002 (Das *et al*., 2004). The fly strains were maintained under standard laboratory conditions (Rearing temperature – RT: 22˚C ± 1˚C 14:10 hours light:dark cycle) at low density on corn-molasses medium. All

experiments were performed on flies that were expanded in large vials for two generations under the same conditions.

Cold Tolerance Phenotype Measurements

For all phenotype measurements, the flies of the F2 generation were collected in 0-to-2 days upon emergence. After a day of recuperation, the flies were anesthetized under brief exposure to CO_2, separated according to sex and placed into small vials with fresh fly food in groups of 10 individuals. The flies were allowed to recuperate another day after sex separation and tested when 4-6 days old. Three replicate vials were used per sex and strain for all phenotyping assays.

Chill Coma Recovery Time

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

Cold Shock Mortality & LTi50

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

Data Analysis

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

Sample Collection and DNA Extraction

Four pools were generated to be used in bulk segregant analysis. For parental pools, 100 females were collected from F2 generations of each founder. For recombinant inbred line pools with extreme phenotypes, the flies from the F2 generation of selected RILs were collected in 0-to-2 days upon emergence. After a day of recuperation, the flies were anesthetized under brief exposure to CO2 and separated according to sex. Single female flies were placed into small vials containing fresh fly food. The 4-6-day old flies were placed on melting ice at 0˚C for 3 hours to induce chill coma. Then, the vials

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

Whole Genome Sequencing

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

Bulk Segregant Analysis

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

Gene Ontology Enrichment Analysis

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

significant GO terms were visualized using the genekitr package (version 1.2.5) (Liu, 2023).

Data Availability

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

Author Contributions

Conceptualization: S.G., V.M.Y; methodology: S.G., V.M.Y.; software: V.M.Y., F.T.K.; validation: S.G., V.M.Y., V.O.; formal analysis: V.M.Y., F.T.K.; investigation: V.M.Y., F.T.K.; resources: S.G.; data curation: V.M.Y., F.T.K; writing – original draft: V.M.Y.; writing – review & editing: S.G., V.M.Y, V.O.; visualization: V.M.Y; supervision: S.G.; project administration: S.G.; 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 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.

References

Andersen, J. L., Findsen, A., & Overgaard, J. (2013). Feeding impairs chill coma recovery in the migratory locust (*Locusta migratoria*). *Journal of Insect Physiology*, *59*(10), 1041–1048. https://doi.org/10.1016/j.jinsphys.2013.07.008

Andersen, J. L., Manenti, T., Sørensen, J. G., MacMillan, H. A., Loeschcke, V., & Overgaard, J. (2015). How to assess *Drosophila* cold tolerance: chill coma temperature and lower lethal temperature are the best predictors of cold distribution limits. *Functional Ecology*, *29*(1), 55–65. https://doi.org/10.1111/1365-2435.12310

Andersen, L. H., Kristensen, T. N., Loeschcke, V., Toft, S., & Mayntz, D. (2010). Protein and carbohydrate composition of larval food affects tolerance to thermal stress and desiccation in adult *Drosophila*

melanogaster. *Journal of Insect Physiology*, *56*(4), 336–340. https://doi.org/10.1016/j.jinsphys.2009.11.006

Ayrinhac, A., Debat, V., Gibert, P., Kister, A. -G., Legout, H., Moreteau, B., Vergilino, R., & David, J. R. (2004). Cold adaptation in geographical populations of *Drosophila melanogaster*: phenotypic plasticity is more important than genetic variability. *Functional Ecology*, *18*(5), 700–706. https://doi.org/10.1111/j.0269-8463.2004.00904.x

Ayrinhac, A., Debat, V., Gibert, P., Kister, A. -G., Legout, H., Moreteau, B., Vergilino, R., & David, J. R. (2004). Cold adaptation in geographical populations of *Drosophila melanogaster*: phenotypic plasticity is more important than genetic variability. *Functional Ecology*, *18*(5), 700–706. https://doi.org/10.1111/j.0269-8463.2004.00904.x

Azad, P., Zhou, D., Zarndt, R., & Haddad, G. G. (2012). Identification of Genes Underlying Hypoxia Tolerance in *Drosophila* by a P-element Screen. *G3 Genes|Genomes|Genetics*, *2*(10), 1169–1178. https://doi.org/10.1534/g3.112.003681

Bannan, B. A., Van Etten, J., Kohler, J. A., Tsoi, Y., Hansen, N. M., Sigmon, S., Fowler, E., Buff, H., Williams, T. S., Ault, J. G., Glaser, R. L., & Korey, C. A. (2008). The *Drosophila* protein palmitoylome: Characterizing palmitoyl-thioesterases and DHHC palmitoyl-transferases. *Fly*, *2*(4), 198–214. https://doi.org/10.4161/fly.6621

Bastide, H., Lange, J. D., Lack, J. B., Yassin, A., & Pool, J. E. (2016). A Variable Genetic Architecture of Melanic Evolution in *Drosophila melanogaster*. *Genetics*, *204*(3), 1307–1319. https://doi.org/10.1534/genetics.116.192492

Baust, J. G., & Lee, R. E. (1987). Multiple stress tolerance in an antarctic terrestrial arthropod: *Belgica antarctica*. *Cryobiology*, *24*(2), 140–147. https://doi.org/10.1016/0011-2240(87)90016-2

Benowitz, K. M., Coleman, J. M., & Matzkin, L. M. (2019). Assessing the Architecture of *Drosophila mojavensis* Locomotor Evolution with Bulk Segregant Analysis. *G3 Genes|Genomes|Genetics*, *9*(5), 1767–1775. https://doi.org/10.1534/g3.119.400036

Bowman, L. L., Kondrateva, E. S., Timofeyev, M. A., & Yampolsky, L. Y. (2018). Temperature gradient affects differentiation of gene expression and SNP allele frequencies in the dominant Lake Baikal zooplankton species. *Molecular Ecology*, *27*(11), 2544–2559. https://doi.org/10.1111/mec.14704

Bryon, A., Kurlovs, A. H., Dermauw, W., Greenhalgh, R., Riga, M., Grbić, M., Tirry, L., Osakabe, M., Vontas, J., Clark, R. M., & Van Leeuwen, T. (2017). Disruption of a horizontally transferred phytoene desaturase abolishes carotenoid accumulation and diapause in *Tetranychus urticae*. *Proceedings of the National Academy of Sciences*, *114*(29). https://doi.org/10.1073/pnas.1706865114

Bubliy, O. A., Riihimaa, A., Norry, F. M., & Loeschcke, V. (2002). Variation in resistance and acclimation to low-temperature stress among three geographical strains of *Drosophila melanogaster*. *Journal of Thermal Biology*, *27*(5), 337–344. https://doi.org/10.1016/S0306-4565(01)00098-5

Chen, S., Yu, M., Chu, X., Li, W., Yin, X., & Chen, L. (2017). Cold-induced retrotransposition of fish LINEs. *Journal of Genetics and Genomics*, *44*(8), 385–394. https://doi.org/10.1016/j.jgg.2017.07.002

Colinet, H., Renault, D., & Roussel, D. (2017). Cold acclimation allows *Drosophila* flies to maintain mitochondrial functioning under cold stress. *Insect Biochemistry and Molecular Biology*, *80*, 52–60. https://doi.org/10.1016/j.ibmb.2016.11.007

Colinet, H., Sciaussat, D., Bozzolan, F., & Bowler, K. (2012). Rapid decline of cold tolerance at young age is associated with expression of stress genes in *Drosophila melanogaster*. *Journal of Experimental Biology*, jeb.076216. https://doi.org/10.1242/jeb.076216

Cottam, D. M., Tucker, J. B., Rogers-Bald, M. M., Mackie, J. B., Macintyre, J., Scarborough, J. A., Ohkura, H., & Milner, M. J. (2006). Non-centrosomal microtubule-organising centres in cold-treated cultured *Drosophila* cells. *Cell Motility*, *63*(2), 88–100. https://doi.org/10.1002/cm.20103

Das, A., Mohanty, S., & Stephan, W. (2004). Inferring the Population Structure and Demography of *Drosophila ananassae* From Multilocus Data. *Genetics*, *168*(4), 1975–1985. https://doi.org/10.1534/genetics.104.031567

David, J. R., Gibert, P., Moreteau, B., Gilchrist, G. W., & Huey, R. B. (2003). The fly that came in from the cold: geographic variation of recovery time from low-temperature exposure in *Drosophila subobscura*. *Functional Ecology*, *17*(4), 425–430. https://doi.org/10.1046/j.1365-2435.2003.00750.x

David, J. R., Gibert, P., Pla, E., Petavy, G., Karan, D., & Moreteau, B. (1998). Cold stress tolerance in *Drosophila*: analysis of chill coma recovery in *D. melanogaster*. *Journal of Thermal Biology*, *23*(5), 291–299. https://doi.org/10.1016/S0306-4565(98)00020-5

Denker, S. P., & Barber, D. L. (2002). Ion transport proteins anchor and regulate the cytoskeleton. *Current Opinion in Cell Biology*, *14*(2), 214–220. https://doi.org/10.1016/S0955-0674(02)00304-6

Des Marteaux, L. E., McKinnon, A. H., Udaka, H., Toxopeus, J., & Sinclair, B. J. (2017). Effects of cold-acclimation on gene expression in Fall field cricket (*Gryllus pennsylvanicus*) ionoregulatory tissues. *BMC Genomics*, *18*(1), 357. https://doi.org/10.1186/s12864-017-3711-9

Edwards, M. D., & Gifford, D. K. (2012). High-resolution genetic mapping with pooled sequencing. *BMC Bioinformatics*, *13*(S6), S8. https://doi.org/10.1186/1471-2105-13-S6-S8

Ehrenreich, I. M., Torabi, N., Jia, Y., Kent, J., Martis, S., Shapiro, J. A., Gresham, D., Caudy, A. A., & Kruglyak, L. (2010). Dissection of genetically complex traits with extremely large pools of yeast segregants. *Nature*, *464*(7291), 1039–1042. https://doi.org/10.1038/nature08923

Gibert, P., Moreteau, B., Pétavy, G., Karan, D., & David, J. R. (2001). Chill-coma tolerance, a major climatic adaptation among *Drosophila* species. *Evolution*, *55*(5), 1063. https://doi.org/10.1554/0014-3820(2001)055[1063:CCTAMC]2.0.CO;2

Gilliland, W. D., Vietti, D. L., Schweppe, N. M., Guo, F., Johnson, T. J., & Hawley, R. S. (2009). Hypoxia Transiently Sequesters Mps1 and Polo to Collagenase-Sensitive Filaments in *Drosophila* Prometaphase Oocytes. *PLoS ONE*, *4*(10), e7544. https://doi.org/10.1371/journal.pone.0007544

Hallas, R., Schiffer, M., & Hoffmann, A. A. (2002). Clinal variation in *Drosophila serrata* for stress resistance and body size. *Genetical Research*, *79*(2), 141–148. https://doi.org/10.1017/S0016672301005523

Hoffmann, A. A., & Watson, M. (1993). Geographical Variation in the Acclimation Responses of *Drosophila* to Temperature Extremes. *The American Naturalist*, *142*, S93–S113. https://www.jstor.org/stable/2462711

Hoffmann, A. A., & Weeks, A. R. (2007). Climatic selection on genes and traits after a 100 year-old invasion: a critical look at the temperate-tropical clines in *Drosophila melanogaster* from eastern Australia. *Genetica*, *129*(2), 133. https://doi.org/10.1007/s10709-006-9010-z

Hoffmann, A. A., Anderson, A., & Hallas, R. (2002). Opposing clines for high and low temperature resistance in *Drosophila melanogaster*. *Ecology Letters*, *5*(5), 614–618. https://doi.org/10.1046/j.1461-0248.2002.00367.x

Jurado, L. A. P., Coloma, A., & Cruces, J. (1999). Identification of a Human Homolog of the *Drosophila* rotated abdomen Gene (POMT1) Encoding a Putative Protein O-Mannosyl-Transferase, and Assignment to Human Chromosome 9q34.1. *Genomics*, *58*(2), 171–180. https://doi.org/10.1006/geno.1999.5819

Kim, M., Robich, R. M., Rinehart, J. P., & Denlinger, D. L. (2006). Upregulation of two actin genes and redistribution of actin during diapause and cold stress in the northern house mosquito, *Culex pipiens*. *Journal of Insect Physiology*, *52*(11–12), 1226–1233. https://doi.org/10.1016/j.jinsphys.2006.09.007

Kobey, R. L., & Montooth, K. (2012). Mortality from desiccation contributes to a genotype-by-temperature interaction for cold survival in *Drosophila melanogaster*. *Journal of Experimental Biology*, jeb.076539. https://doi.org/10.1242/jeb.076539

Königer, A., & Grath, S. (2018). Transcriptome Analysis Reveals Candidate Genes for Cold Tolerance in *Drosophila ananassae*. *Genes*, *9*(12), 624. https://doi.org/10.3390/genes9120624

Königer, A., Arif, S., & Grath, S. (2019). Three Quantitative Trait Loci Explain More than 60% of Variation for Chill Coma Recovery Time in a Natural Population of *Drosophila ananassae*. *G3 Genes|Genomes|Genetics*, *9*(11), 3715–3725. https://doi.org/10.1534/g3.119.400453

Kurlovs, A. H., Snoeck, S., Kosterlitz, O., Van Leeuwen, T., & Clark, R. M. (2019). Trait mapping in diverse arthropods by bulked segregant analysis. *Current Opinion in Insect Science*, *36*, 57–65. https://doi.org/10.1016/j.cois.2019.08.004

Linder, M. E., & Deschenes, R. J. (2007). Palmitoylation: policing protein stability and traffic. *Nature Reviews Molecular Cell Biology*, *8*(1), 74–84. https://doi.org/10.1038/nrm2084

Liu, Y., & Li, G. (2023). Empowering biologists to decode omics data: the Genekitr R package and web server. *BMC Bioinformatics*, *24*(1), 214. https://doi.org/10.1186/s12859-023-05342-9

Ma, X., Zhu, M., Liu, W., Li, J., Liao, Y., Liu, D., Jin, M., Fu, C., & Wang, F. (2023). Bulk segregant analysis coupled with transcriptomics and metabolomics revealed key regulators of bacterial leaf blight resistance in rice. *BMC Plant Biology*, *23*(1), 332. https://doi.org/10.1186/s12870-023-04347-z

MacMillan, H. A., Andersen, J. L., Davies, S. A., & Overgaard, J. (2015). The capacity to maintain ion and water homeostasis underlies interspecific variation in *Drosophila* cold tolerance. *Scientific Reports*, *5*(1), 18607. https://doi.org/10.1038/srep18607

MacMillan, H. A., Knee, J. M., Dennis, A. B., Udaka, H., Marshall, K. E., Merritt, T. J. S., & Sinclair, B. J. (2016). Cold acclimation wholly reorganizes the *Drosophila melanogaster* transcriptome and metabolome. *Scientific Reports*, *6*(1), 28999. https://doi.org/10.1038/srep28999

MacMillan, H. A., Yerushalmi, G. Y., Jonusaite, S., Kelly, S. P., & Donini, A. (2017). Thermal acclimation mitigates cold-induced paracellular leak from the *Drosophila* gut. *Scientific Reports*, *7*(1), 8807. https://doi.org/10.1038/s41598-017-08926-7

Magwene, P. M., Willis, J. H., & Kelly, J. K. (2011). The Statistics of Bulk Segregant Analysis Using Next Generation Sequencing. *PLoS Computational Biology*, *7*(11), e1002255. https://doi.org/10.1371/journal.pcbi.1002255

Mansfeld, B. N., & Grumet, R. (2018). QTLseqr: An R Package for Bulk Segregant Analysis with Next-Generation Sequencing. *The Plant Genome*, *11*(2), 180006. https://doi.org/10.3835/plantgenome2018.01.0006

Michelmore, R. W., Paran, I., & Kesseli, R. V. (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Proceedings of the National Academy of Sciences*, *88*(21), 9828–9832. https://doi.org/10.1073/pnas.88.21.9828

Nilson, T. L., Sinclair, B. J., & Roberts, S. P. (2006). The effects of carbon dioxide anesthesia and anoxia on rapid cold-hardening and chill coma recovery in *Drosophila melanogaster*. *Journal of Insect Physiology*, *52*(10), 1027–1033. https://doi.org/10.1016/j.jinsphys.2006.07.001

Overgaard, J., & MacMillan, H. A. (2017). The Integrative Physiology of Insect Chill Tolerance. *Annual Review of Physiology*, *79*(1), 187–208. https://doi.org/10.1146/annurev-physiol-022516-034142

Overgaard, J., Gerber, L., & Andersen, M. K. (2021). Osmoregulatory capacity at low temperature is critical for insect cold tolerance. *Current Opinion in Insect Science*, *47*, 38–45. https://doi.org/10.1016/j.cois.2021.02.015

Parker, D. J., Envall, T., Ritchie, M. G., & Kankare, M. (2021). Sex-specific responses to cold in a very cold-tolerant, northern *Drosophila* species. *Heredity*, *126*(4), 695–705. https://doi.org/10.1038/s41437-020-00398-2

Parker, D. J., Vesala, L., Ritchie, M. G., Laiho, A., Hoikkala, A., & Kankare, M. (2015). How consistent are the transcriptome changes associated with cold acclimation in two species of the *Drosophila virilis* group? *Heredity*, *115*(1), 13–21. https://doi.org/10.1038/hdy.2015.6

Pool, J. E. (2016). Genetic Mapping by Bulk Segregant Analysis in *Drosophila*: Experimental Design and Simulation-Based Inference. *Genetics*, *204*(3), 1295–1306. https://doi.org/10.1534/genetics.116.192484

R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.URL https://www.R-project.org/

Ring, R. A. (1982). Freezing-tolerant insects with low supercooling points. *Comparative Biochemistry and Physiology Part A: Physiology*, *73*(4), 605–612. https://doi.org/10.1016/0300-9629(82)90267-5

Schnorrer, F., Schönbauer, C., Langer, C. C. H., Dietzl, G., Novatchkova, M., Schernhuber, K., Fellner, M., Azaryan, A., Radolf, M., Stark, A., Keleman, K., & Dickson, B. J. (2010). Systematic genetic analysis of muscle morphogenesis and function in *Drosophila*. *Nature*, 464(7286), 287–291. https://doi.org/10.1038/nature08799

Sinclair, B.J. (1999). Insect cold tolerance: How many kinds of frozen? *European Journal of Entomology*, *96*(2), 157-164

Sisodia, S., & Singh, B. N. (2010). Influence of developmental temperature on cold shock and chill coma recovery in *Drosophila ananassae*: Acclimation and latitudinal variations among Indian populations. *Journal of Thermal Biology*, *35*(3), 117–124. https://doi.org/10.1016/j.jtherbio.2010.01.001

Sisodia, S., & Singh, B. N. (2012). Experimental Evidence for Nutrition Regulated Stress Resistance in *Drosophila ananassae*. *PLoS ONE*, *7*(10), e46131. https://doi.org/10.1371/journal.pone.0046131

Snoeck, S., Kurlovs, A. H., Bajda, S., Feyereisen, R., Greenhalgh, R., Villacis-Perez, E., Kosterlitz, O., Dermauw, W., Clark, R. M., & Van Leeuwen, T. (2019). High-resolution QTL mapping in Tetranychus

urticae reveals acaricide-specific responses and common target-site resistance after selection by different METI-I acaricides. *Insect Biochemistry and Molecular Biology*, *110*, 19–33. https://doi.org/10.1016/j.ibmb.2019.04.011

Strachan, L. A., Tarnowski-Garner, H. E., Marshall, K. E., & Sinclair, B. J. (2011). The Evolution of Cold Tolerance in *Drosophila* Larvae. *Physiological and Biochemical Zoology*, *84*(1), 43–53. https://doi.org/10.1086/657147

Takagi, H., Abe, A., Yoshida, K., Kosugi, S., Natsume, S., Mitsuoka, C., Uemura, A., Utsushi, H., Tamiru, M., Takuno, S., Innan, H., Cano, L. M., Kamoun, S., & Terauchi, R. (2013). QTL -seq: rapid mapping of quantitative trait loci in rice by whole genome resequencing of DNA from two bulked populations. *The Plant Journal*, *74*(1), 174–183. https://doi.org/10.1111/tpj.12105

Teets, N. M., & Denlinger, D. L. (2013). Physiological mechanisms of seasonal and rapid cold-hardening in insects. *Physiological Entomology*, *38*(2), 105–116. https://doi.org/10.1111/phen.12019

Teets, N. M., Elnitsky, M. A., Benoit, J. B., Lopez-Martinez, G., Denlinger, D. L., & Lee, R. E. (2008). Rapid cold-hardening in larvae of the Antarctic midge *Belgica antarctica:* cellular cold-sensing and a role for calcium. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *294*(6), R1938–R1946. https://doi.org/10.1152/ajpregu.00459.2007

Teets, N. M., Kawarasaki, Y., Lee, R. E., & Denlinger, D. L. (2012). Energetic consequences of repeated and prolonged dehydration in the Antarctic midge, Belgica antarctica. *Journal of Insect Physiology*, *58*(4), 498–505. https://doi.org/10.1016/j.jinsphys.2011.11.011

Tobari, Y. N. (ed.), *Drosophila ananassae* - Genetical and Biological Aspects, Japan Scientific Societies Press, Tokyo, 1993.

Volk, T. (1992). A new member of the spectrin superfamily may participate in the formation of embryonic muscle attachments in Drosophila. Development 116(3): 721–730.

Von Heckel, K., Stephan, W., & Hutter, S. (2016). Canalization of gene expression is a major signature of regulatory cold adaptation in temperate *Drosophila melanogaster*. *BMC Genomics*, *17*(1), 574. https://doi.org/10.1186/s12864-016-2866-0

Vos, R. A., Van Der Veen-van Wijk, C. A. M., Schranz, M. E., Vrieling, K., Klinkhamer, P. G. L., & Lens, F. (2022). Refining bulk segregant analyses: ontology-mediated discovery of flowering time genes in Brassica oleracea. *Plant Methods*, *18*(1), 92. https://doi.org/10.1186/s13007-022-00921-y

Wang, J., Gao, G., Zhang, R., Dai, L., & Chen, H. (2017). Metabolism and cold tolerance of C hinese white pine beetle *Dendroctonus armandi* (C oleoptera: C urculionidae: S colytinae) during the overwintering period. *Agricultural and Forest Entomology*, *19*(1), 10–22. https://doi.org/10.1111/afe.12176

Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., Feng, T., Zhou, L., Tang, W., Zhan, L., Fu, X., Liu, S., Bo, X., & Yu, G. (2021). clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *The Innovation*, *2*(3), 100141. https://doi.org/10.1016/j.xinn.2021.100141

Wybouw, N., Kosterlitz, O., Kurlovs, A. H., Bajda, S., Greenhalgh, R., Snoeck, S., Bui, H., Bryon, A., Dermauw, W., Van Leeuwen, T., & Clark, R. M. (2019). Long-Term Population Studies Uncover the Genome Structure and Genetic Basis of Xenobiotic and Host Plant Adaptation in the Herbivore *Tetranychus urticae*. *Genetics*, *211*(4), 1409–1427. https://doi.org/10.1534/genetics.118.301803

Yılmaz, V. M., Bao, Z., & Grath, S. (2025). Navigating the Cold: Integrative Transcriptome Sequencing Approach Reveals Ionoregulatory and Whole-Body Responses to Cold Acclimation in *Drosophila*

ananassae. *Accepted for publication in Genome Biology and Evolution, available on bioRxiv under doi: 10.1101/2024.07.18.604044*

Yılmaz, V. M., Ramnarine, T. J. S., Königer, A., Mussgnug, S., & Grath, S. (2023). Tropical super flies: Integrating *Cas9* into *Drosophila ananassae* and its phenotypic effects. *Journal of Insect Physiology*, *147*, 104516. https://doi.org/10.1016/j.jinsphys.2023.104516

Figures

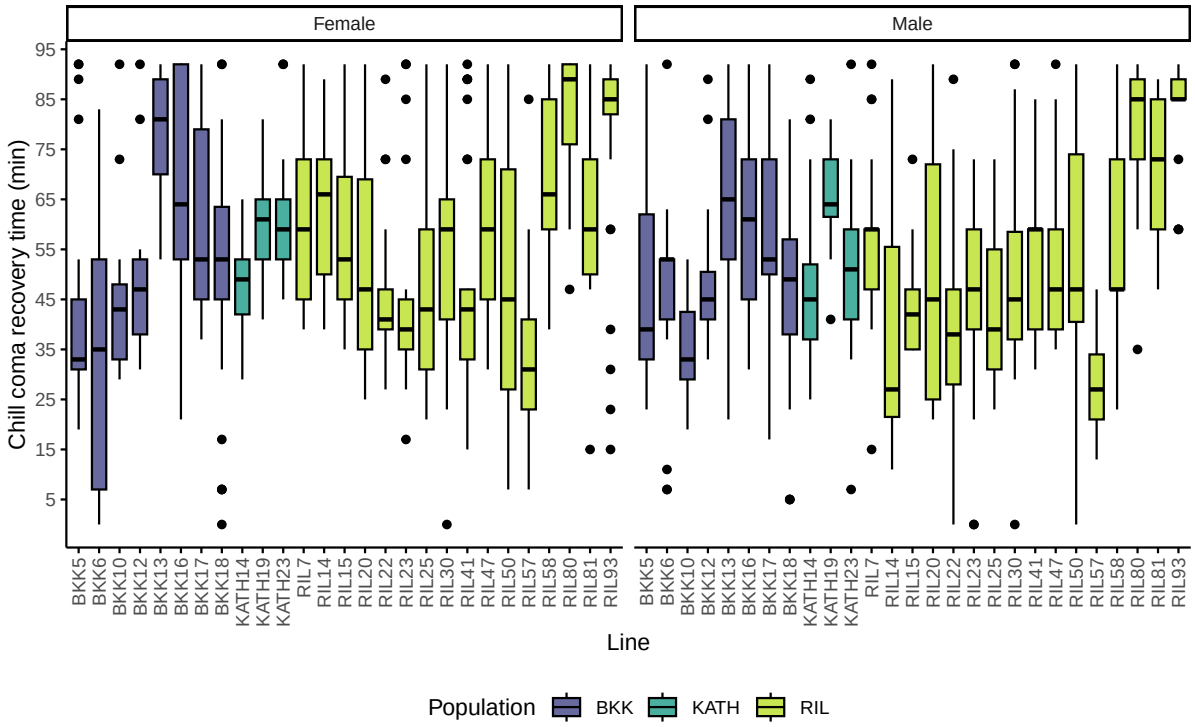

Figure 1: Chill coma recovery time (in minutes) of iso-female and recombinant inbred lines in female (left panel) and male (right panel) flies. The colors indicate the origin of the iso-female lines: Bangkok (purple), Kathmandu (blue), recombinant inbred lines (green). The black dots indicate outliers.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

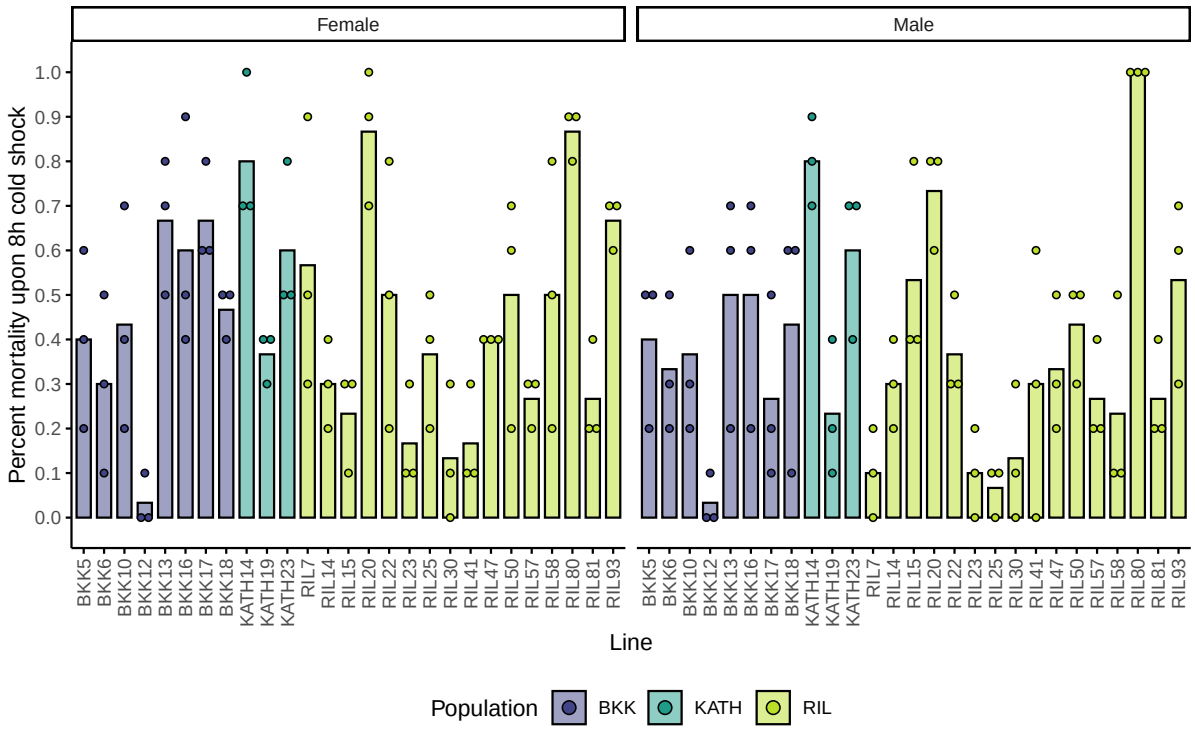

Figure 2: Percent mortality of iso-female and recombinant inbred lines in female (left panel) and male (right panel) flies measured after 8h cold shock followed by 2h recovery. The colors indicate the origin of the iso-female lines: Bangkok (purple), Kathmandu (blue), recombinant inbred lines (green).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

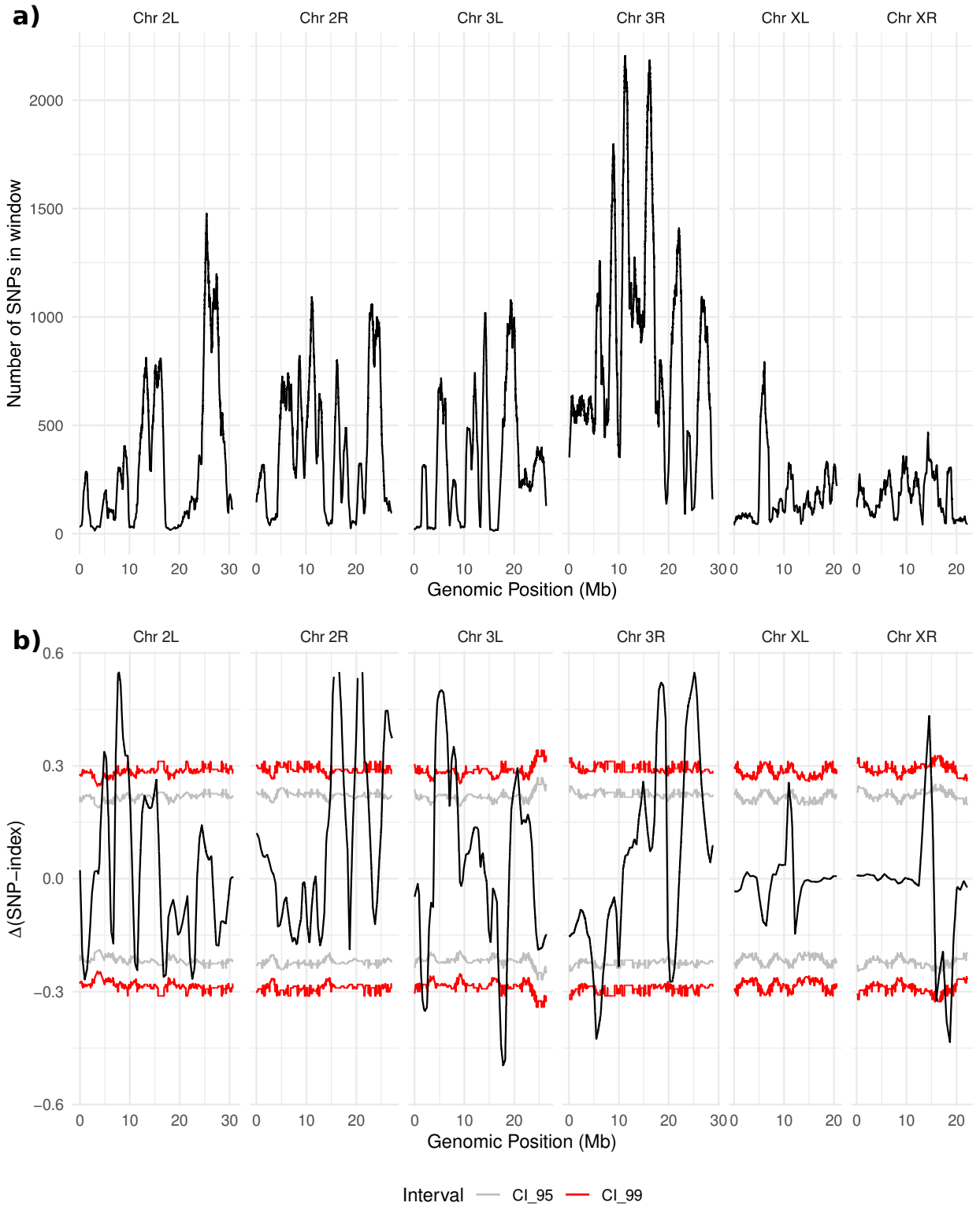

Figure 3: QTL-Seq analysis. **A)** SNP density across genome within 1 Mb windows. **B)** ΔSNP-index plot across the genome. Confidence intervals (CI) shown in grey (95%) and red (99%). Significant QTLs were determined using 99% CI.

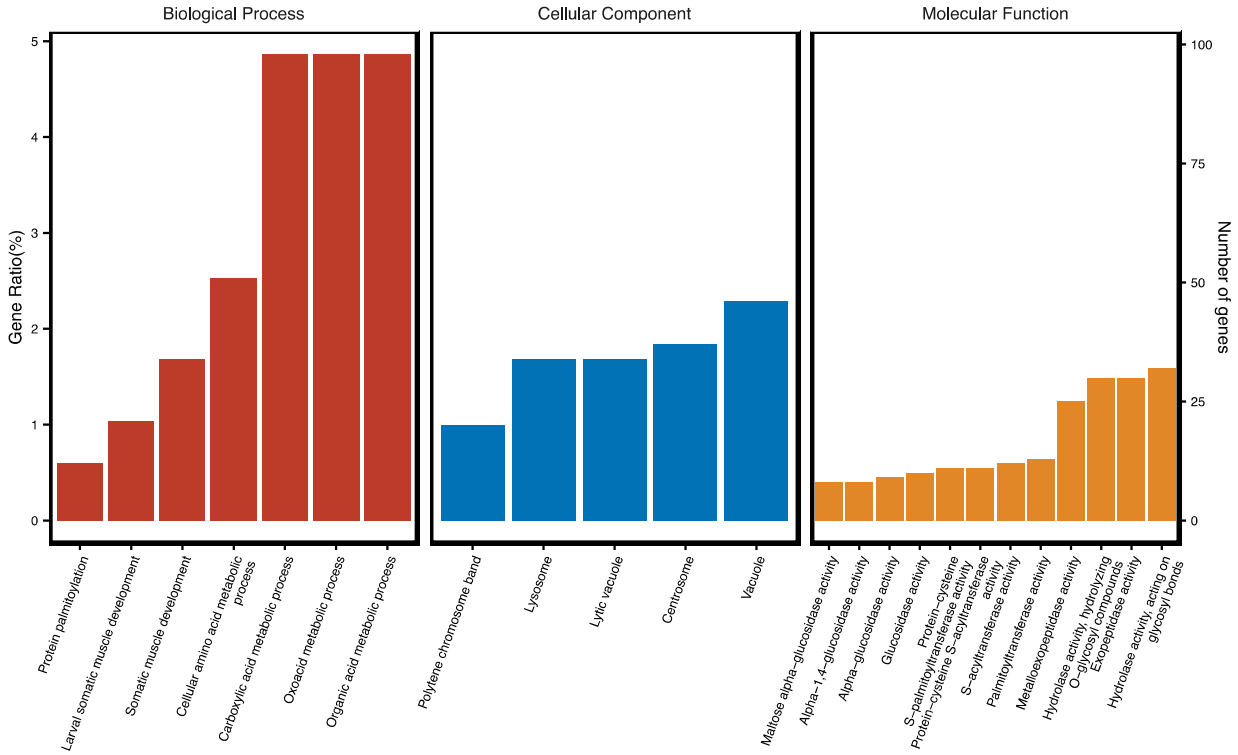

Figure 4: Gene ontology enrichment analysis for genes within the significant QTL regions. Biological processes shown in red, cellular components shown in blue and molecular functions shown in yellow.

Supplementary Material

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

Vera Miyase Yılmaz, Vincent Ohlhauser, Fikri Tuğberk Kara and Sonja Grath

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 bold.

	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 bold.

	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 bold.

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

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	-

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

Supplementary Figures

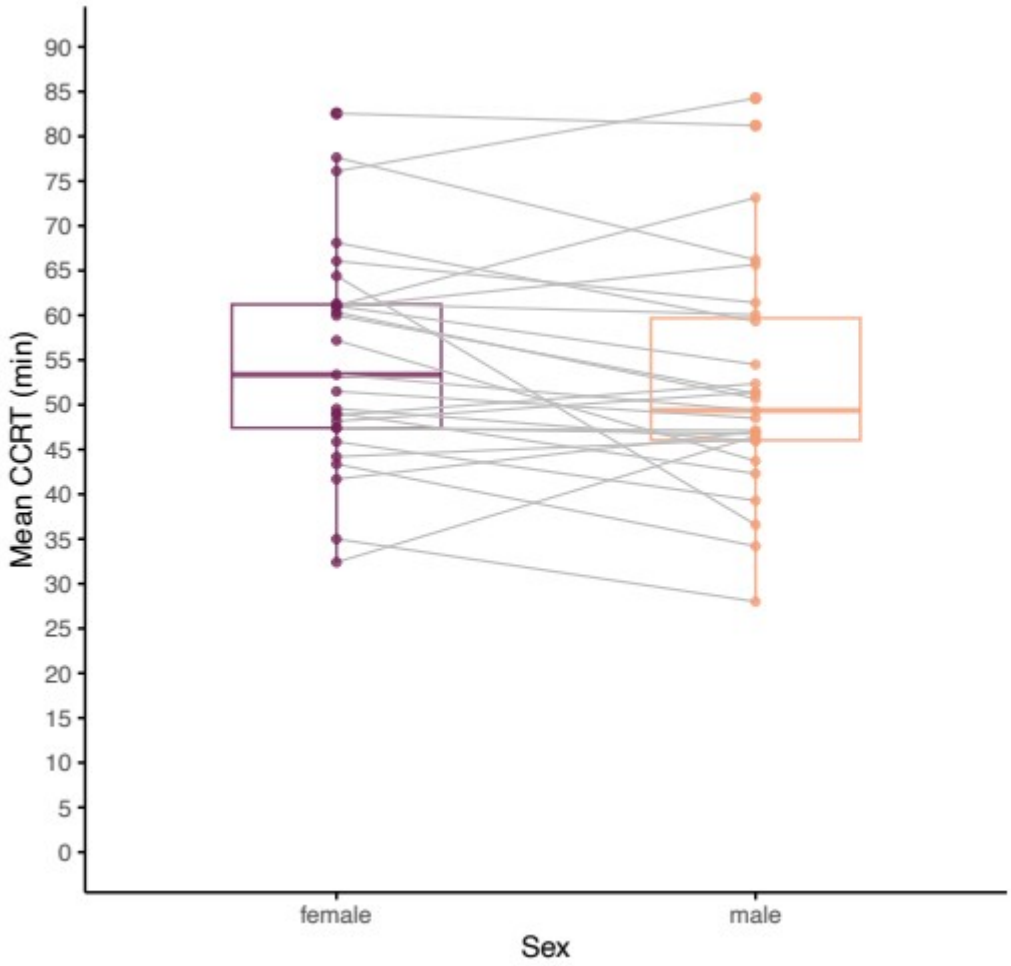

Figure S1: Boxplot for mean CCRT. Females (purple) and males (orange) of the strains are connected with grey lines.

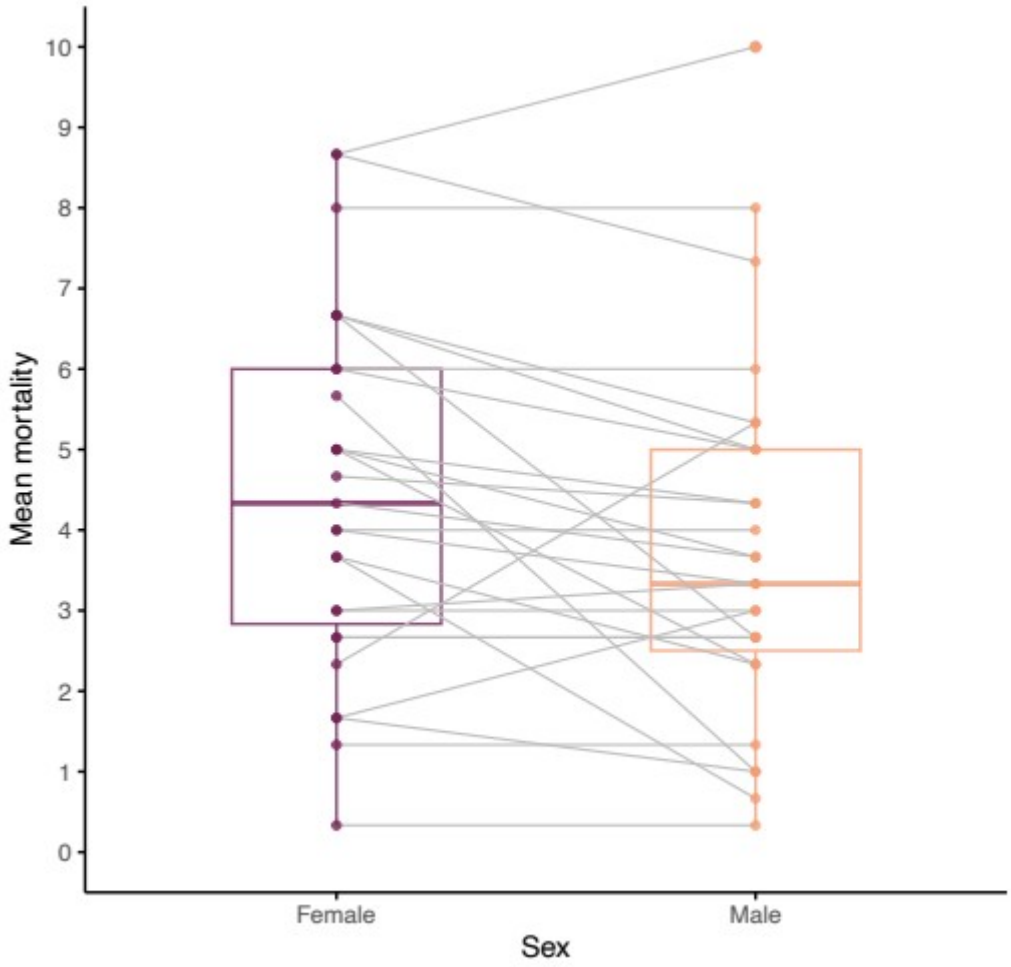

Figure S2: Boxplot for mean mortality upon 8-hour cold shock. Females (purple) and males (orange) of the strains are connected with grey lines.

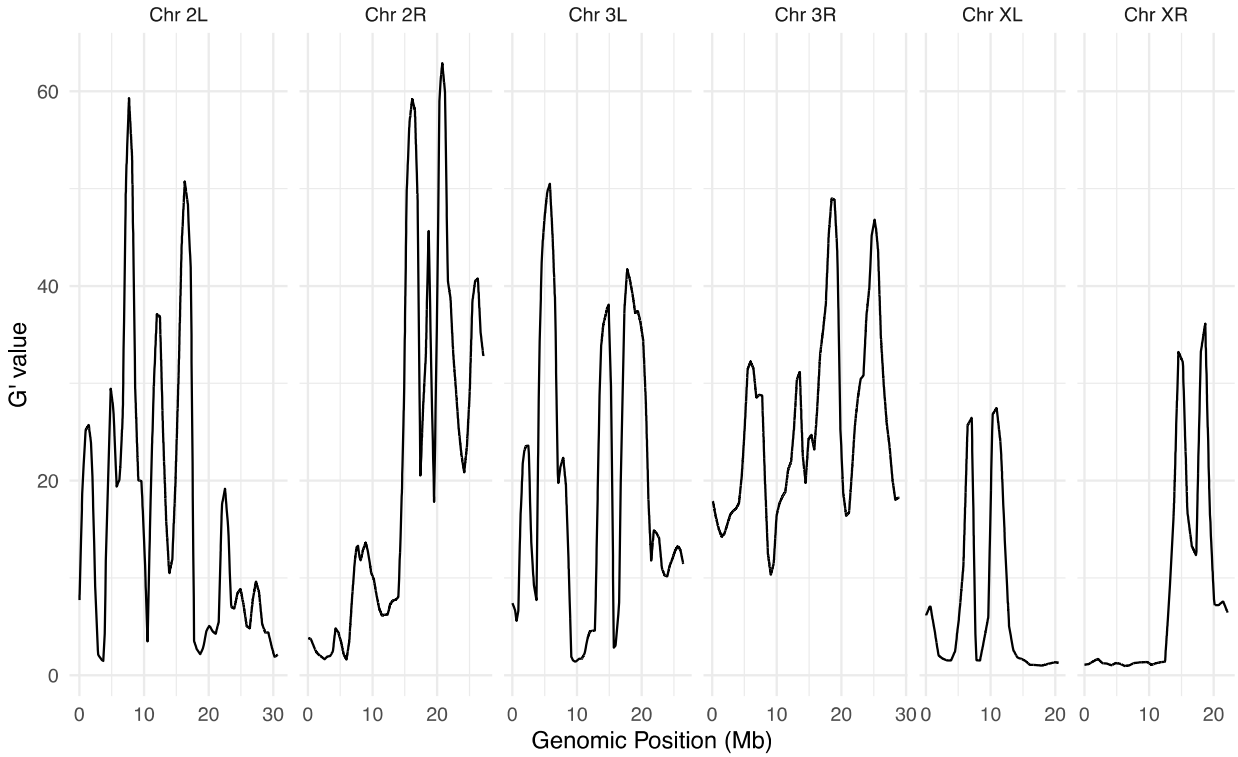

Figure S3: Association plot for each chromosome arm: sliding window (1 Mb) G' analysis. x-axis indicates genomic position on each chromosome arm, y-axis indicates G' value. FDR = 0.01 (not shown).

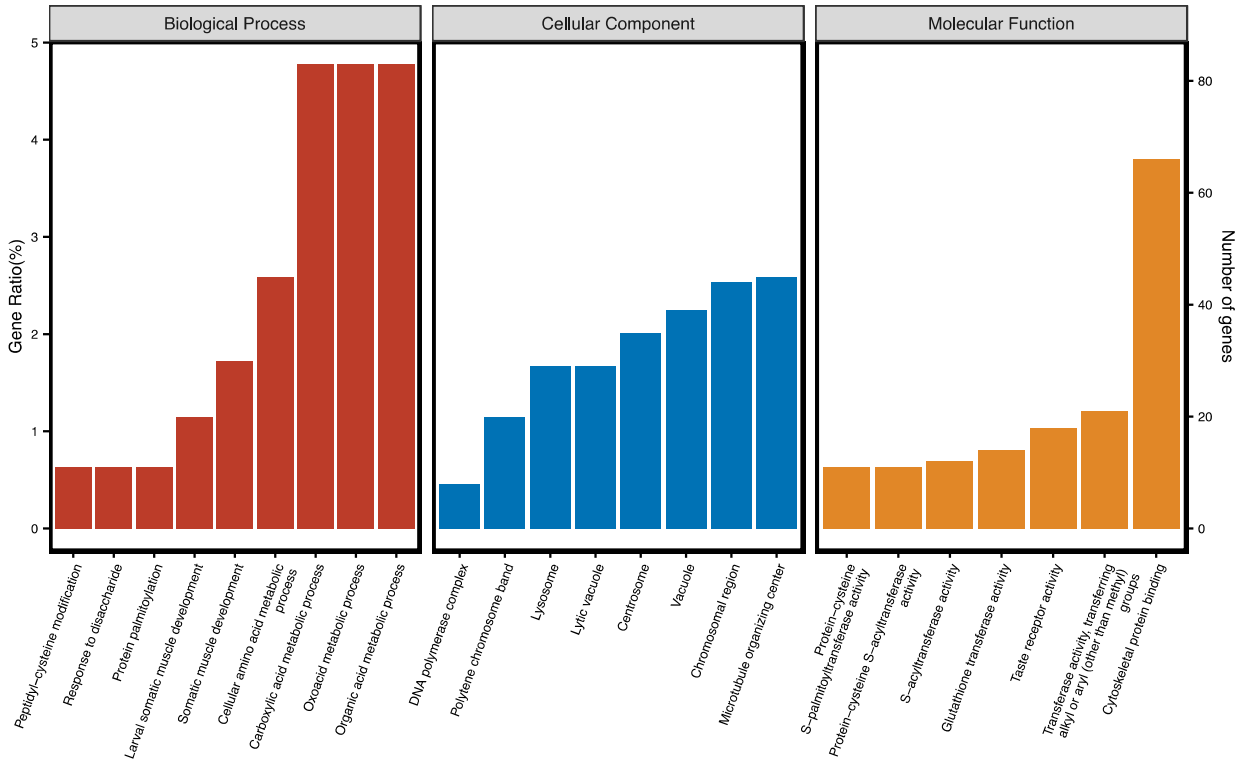

Figure S4: Gene ontology enrichment analysis for genes within the significant QTL regions that had positive ΔSNP-index values. Biological processes shown in red, cellular components shown in blue and molecular functions shown in yellow.

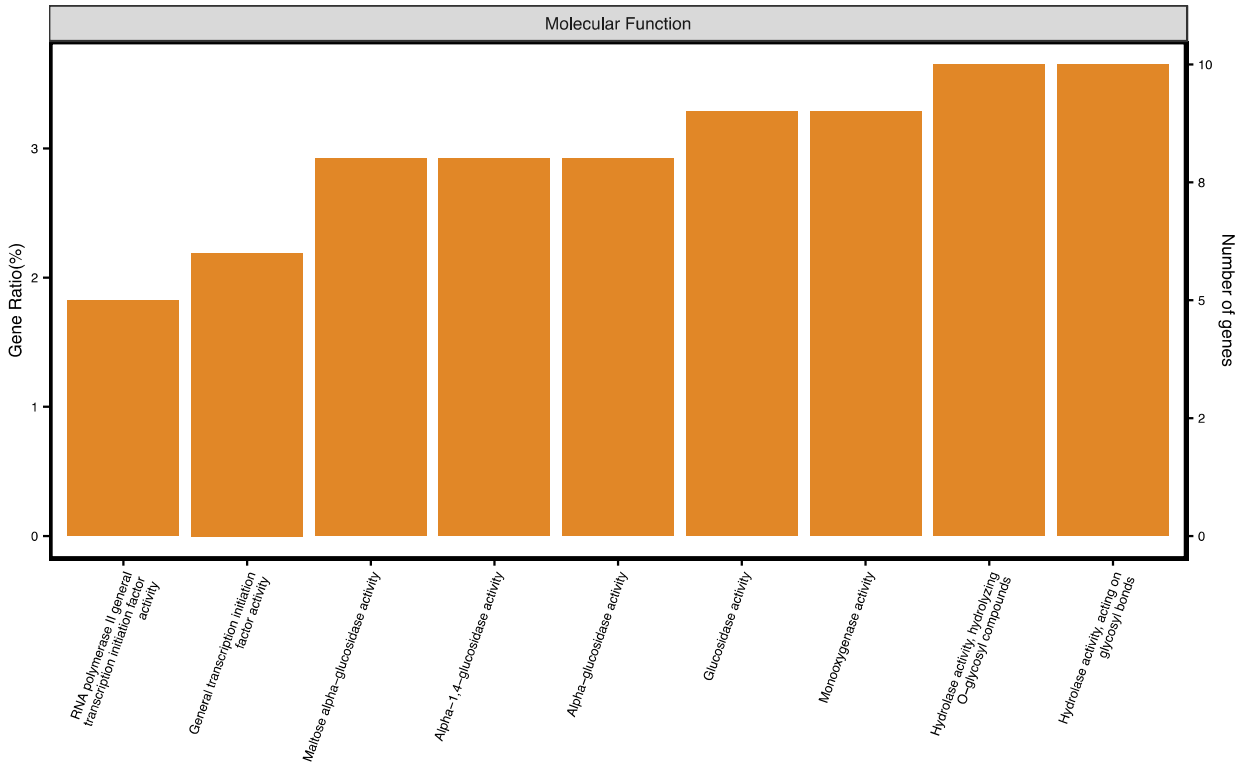

Figure S5: Gene ontology enrichment analysis for genes within the significant QTL regions that had negative ΔSNP-index values. Molecular functions shown in yellow.

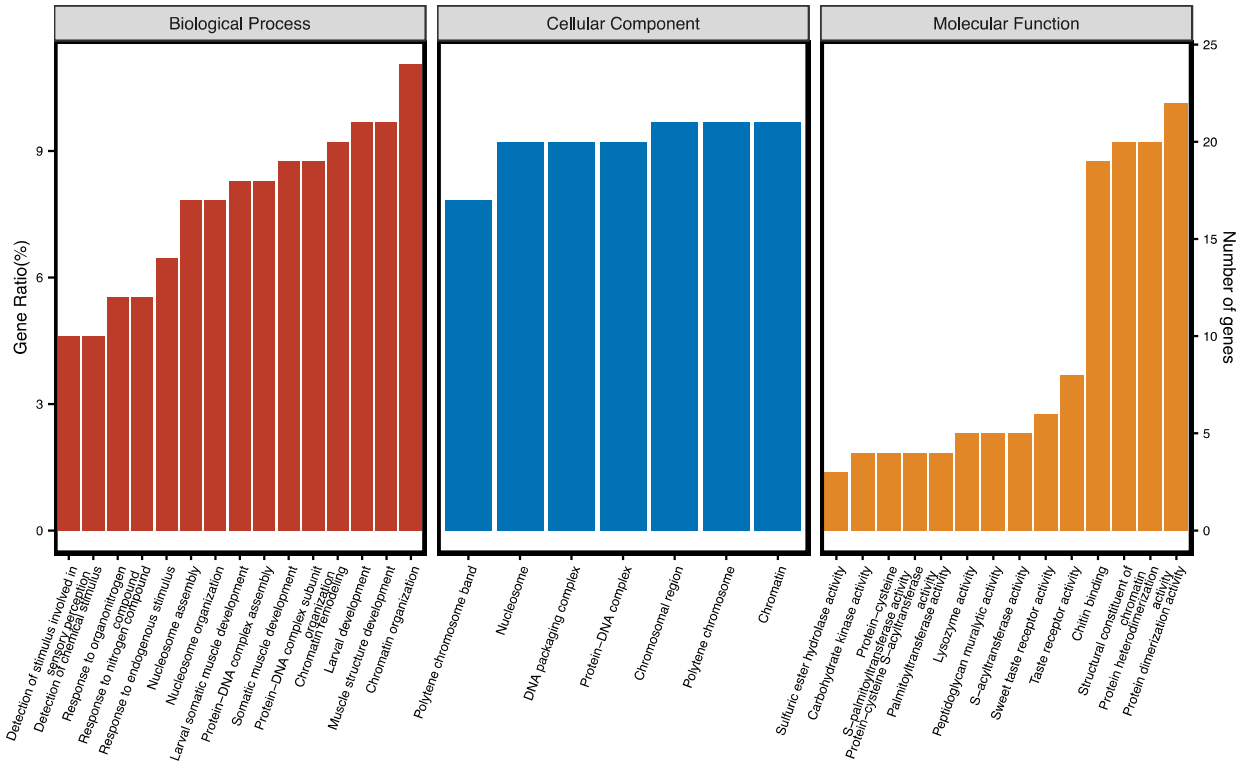

Figure S6: Gene ontology enrichment analysis for genes within the 682-kb region shared by the Δ(SNP-index) and G' analyses. Biological processes shown in red, cellular components shown in blue and molecular functions shown in yellow.