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Navigating the Cold: Integrative Transcriptome Sequencing Approach Reveals Ionoregulatory and Whole-Body Responses to Cold Acclimation in *Drosophila ananassae*

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Running title: Adaptation to cold in *Drosophila*

Abstract

Understanding how species adapt to changing environments is a major goal in evolutionary biology and can elucidate the impact of climate change. Climate imposes inevitable effects on the geographical distribution of insects as their body temperature primarily depends on the environment. The vinegar fly *Drosophila ananassae* expanded from its tropical ancestral range to more temperate regions, which requires adaptation to colder temperatures. Transcriptome and genome-wide association studies focusing on the ancestral-range population identified the targets of selection related to ionoregulatory tissues. However, how cosmopolitan *D. ananassae* adapted to colder environments, where low temperatures last longer, is still unknown. Here, we present a study on the effect of long-term cold exposure on *D. ananassae*, examining the gene expression variation in the whole body and the ionoregulatory tissues, namely the hindgut and the Malpighian tubule. To elucidate molecular mechanisms of cold adaptation during species expansion, we included cold-tolerant and cold-sensitive strains from the ancestral species range and cold-tolerant strains from the derived species range. We show that cold acclimation improves cold tolerance and results in differential expression of more than half of the transcriptome in the ionoregulatory tissues and the whole body. Notably, we provide complementary insight into molecular processes at four levels: strains, populations, phenotypes, and tissues. By determining the biochemical pathways of phenotypic plasticity underlying cold tolerance, our results enhance our understanding of how environmental changes affect thermal adaptation in natural populations.

Keywords: thermoregulation, ectothermy, metabolic cold adaptation, gene expression, phenotypic plasticity, ionoregulatory evolution, acclimation

Introduction

Temperature is a major factor influencing how species adapt to changing environments and colonize new ecosystems. How animals generally cope with thermal stressors and which genes are specifically responsible for cold adaptation has been a topic of intense research for decades in molecular and evolutionary biology. Recent developments in high-throughput sequencing methods allow insights into molecular mechanisms at the whole transcriptome and genome level, but integrating the complementary information of various experimental designs remains challenging. For example, studies on small insects often rely on either systemic or tissue-specific methodologies or single phenotypes, resulting in incomplete conclusions (Andersen *et al.*, 2015; Hoedjes *et al.*, 2024). Therefore, integrating different experimental designs and phenotypes to study transcriptional responses to cold is critical to uncovering mechanisms underlying physiological adaptation. Elucidating the mechanisms that underlie adaptation to changing environments in natural populations and integrating different approaches can help us understand which species prove to be successful in the future and have potential applications for conservation, agriculture, and pest management.

Many insects evolved and diversified in warm climates (Bale & Hayward, 2010). Nevertheless, insects have developed various strategies to cope with thermal stress and to successfully expand their thermal and habitat ranges (Overgaard & MacMillan, 2017). Some insects even tolerate freezing and remain active, such as the Antarctic midge *Belgica antarctica* (Teets *et al.*, 2012) or the alpine beetle *Pytho deplanatus* (Ring, 1982). However, a wide range of insect species suffer from chilling injuries and eventually die when they are exposed to cold. This chill-susceptibility has been reported for several orders of insects (Overgaard & MacMillan, 2017). Chill-susceptible species include some of great economic and ecological importance, such as the honeybee *Apis*

60 *mellifera* (Free & Spencer-Booth, 1960), but also agricultural pests, for instance, the migratory
61 locust *Locusta migratoria* (Bayley *et al.*, 2018) and disease vectors such as mosquitoes (Reinhold
62 *et al*., 2018).

63 The physiology of cold stress resistance in insects is highly complex. When insects are exposed to
64 cold, their ion and water homeostasis is disrupted (MacMillan *et al.*, 2015). Additionally,
65 transcriptomic and metabolic profiles are changed (MacMillan *et al.*, 2016). Along with these
66 systemic changes, chilling injuries affect the body at the cell level as they cause phase changes in
67 the cell membrane, resulting in a stiff membrane with decreased permeability (Steponkus, 1984;
68 Koštál, 2010; Drobnis *et al.*, 1993). Further, cold exposure results in an imbalance of passive
69 diffusion and active ion transport (MacMillan & Sinclair, 2011; Koštál *et al.*, 2004; Zachariassen
70 *et al.*, 2004). An excess of potassium ions in the extracellular space leads to depolarization of the
71 membrane (Findsen *et al.*, 2014; Hoyle, 1953), eventually activating the voltage-gated calcium
72 channels (Salkoff & Wyman, 1983; Findsen *et al.*, 2016). The cellular influx of calcium ions
73 through voltage-gated channels activates apoptotic and necrotic pathways, leading to chill injury
74 (Koštál *et al.*, 2004, 2007, 2016; Zachariassen *et al.*, 2004; MacMillan *et al.*, 2015, 2016).
75 Consequently, the regulation of ion and water homeostasis has been identified as a key
76 physiological feature for cold tolerance in insects with specifically the ionoregulatory tissues being
77 essential for maintaining the water-ion balance in an insect body (Andersen *et al.*, 2017; Andersen
78 & Overgaard, 2020; Des Marteaux *et al.*, 2018; Gerber & Overgaard, 2018; MacMillan *et al.*,
79 2015, 2016, 2017; Yerushalmi *et al.*, 2018). Despite several in-depth studies aiming to resolve this
80 complexity, it remains challenging to identify and functionally characterize specific genes and
81 genetic elements that contribute to the diversity of cold stress-resistant phenotypes (Udaka *et al.*,

82 2010; von Heckel *et al.*, 2016; MacMillan *et al.*, 2016). With their worldwide distribution,
83 Drosophilids are excellent models for studying these questions.

84 Flies from the genus *Drosophila* suffer from impaired synaptic transmission and muscular failure
85 when they are exposed to cold; in other words, they lose their neuromuscular coordination and
86 enter a comatose condition referred to as chill coma in which they are not able to move, feed, mate
87 or escape predators (Kelty *et al.*, 1996; MacMillan & Sinclair, 2011; Findsen *et al.*, 2014).
88 However, flies can recover from such a coma given that time and temperature of exposure to cold
89 do not exceed certain thresholds. The time the fly needs for recovery from a cold-induced coma
90 (chill coma recovery time, CCRT) is widely used to assess the cold hardiness of insects in the
91 laboratory and serves as a predictor for the climatic condition at the geographical sampling site of
92 a particular species (Gibert *et al.*, 2001). Further, CCRT follows latitudinal clines within
93 *Drosophila* species in Australia (Hoffmann *et al.*, 2002; Hoffmann & Weeks, 2007; Hallas *et al.*,
94 2002), Asia (Sisodia & Singh, 2010), Africa and Europe (David *et al.*, 2003). Cold tolerance in
95 Drosophilids is polygenic. Despite this complexity, variation in gene expression of only a few loci
96 is associated with cold tolerance in subtropical populations of *Drosophila ananassae* (Königer &
97 Grath, 2018), and variation of just three genomic regions explained more than 60% of the
98 phenotypic variation between cold-tolerant and cold-sensitive strains (Königer *et al.*, 2019).
99 Recently, we provided Cas9-integrated fly strains that facilitate the use of the CRISPR-Cas9
100 system in this species and allow functional characterization of individual candidate loci (Yılmaz
101 *et al*., 2023). In sum, *D. ananassae* has been established as a promising model for studying the
102 genetic basis of cold tolerance.

103 Previous studies mostly neglected the potential of integrating two specific aspects to better inform
104 about cold tolerance in insects. First, how insects respond to low temperatures can be plastic: the

105 ability to tolerate cold and the genes responsible for the survival can change depending on prior
106 exposure to cold. Such acclimation has been shown to alleviate the adverse effects of cold and can
107 increase cold tolerance (Bubliy *et al.*, 2002; Enriquez & Colinet, 2019; Hoffmann & Watson, 1993;
108 MacMillan *et al.*, 2016, 2017; Watson & Hoffmann, 1996; Yılmaz *et al.*, 2023). Cold acclimation
109 triggers substantial changes in the transcriptional response to cold in *Drosophila suzukii* (Enriquez
110 & Colinet 2019). It reorganizes the transcriptome and metabolome of whole animals (MacMillan
111 *et al.*, 2016) and the gut transcriptome in *Drosophila melanogaster* (MacMillan *et al.*, 2017).
112 Second, conclusions on potentially ecologically relevant adaptations should not rely on a single
113 phenotype (Andersen *et al.*, 2015). Consequently, incorporating distinct cold tolerance phenotypes
114 and systemic and ionoregulatory tissue-specific transcriptome data will allow pinpointing cold-
115 related physiological adaptations.

116 In this study, we use iso-female lines collected from a population of ancestral origin in Bangkok,
117 Thailand (from here on mentioned as ancestral population) and a derived population originating
118 from Kathmandu, Nepal (Das *et al.*, 2004). We examined the transcriptional response to cold
119 acclimation in both the ancestral and the derived populations to shed light on the genetic
120 mechanisms underlying cold tolerance. In addition, we integrated the original results of this study
121 with previously gained insight into the distinct phenotype of cold shock. To this end, we contrasted
122 the transcriptional response to cold at four levels: (1) population (ancestral vs. derived), (2) strain
123 (cold-sensitive vs. cold-tolerant), (3) tissue (whole-body vs. ionoregulatory tissues, Malpighian
124 tubules and hindgut), and (4) phenotype (cold acclimation vs. cold shock). We find empirical
125 evidence for the influence of population and tissue on gene expression variation upon cold stress.
126 Using an RNA sequencing approach that combined systemic with tissue-specific transcriptomes,

127 we show that cold acclimation improves cold tolerance and results in more than 50% of the
128 transcriptome being differentially expressed in the whole body the ionoregulatory tissues.

129 Our results demonstrate that an RNA sequencing strategy combining a systemic overview with
130 tissue-specific data can be a cost-effective and informative way to elucidate genes that have major
131 effects on a crucial adaptation-related trait, namely, thermoregulation.

Results

Complementary elucidation of transcriptional response to cold acclimation with systemic whole body and tissue-specific data

Flies from ancestral and derived populations were previously described as having different sensitivity to cold shock as determined by CCRT (Königer & Grath, 2018). Additionally, preliminary tests revealed that acclimation to cold improves the tolerance and decreases the chill coma recovery time (see Supplementary Text). To receive an informative overview of the transcriptional response to cold acclimation in *D. ananassae*, we combined high-throughput RNA sequencing of the whole body (wb), as well as of two ionoregulatory tissues, Malpighian tubules (Mt) and hindguts (hg). We obtained RNA sequencing data from male flies upon 4-day acclimation at 10°C. In the whole body and hindgut samples, 54% of all expressed genes were differentially expressed in response to cold acclimation, and the percentage of DE genes in the Malpighian tubules was 50% (Table 1). The principal component analysis revealed that the samples clustered according to the tissue RNA was extracted from, whereas no separation according to the treatment was observed (Figure 1A). The first principal component explained 68% of the variance, separating the ionoregulatory tissues from the whole-body samples. The second principal component explained 19% of the variance, separating the two ionoregulatory tissues. Though pairwise comparisons revealed that 2952 differentially expressed genes were shared between sample types, a considerable number of genes (ranging from 505 genes that were explicitly up-regulated in Malpighian tubules to 1436 genes that were down-regulated explicitly in the whole body) were specifically up- or down-regulated only in the individual tissues (Figure 1B-C). Notably, these genes were enriched in distinct GO categories (Supplementary Table 3-4). These

154 results suggest that the combination of systemic and informed tissue-specific RNA sequencing
155 data allows for complementary conclusions on cold tolerance in *Drosophila*.

156 **Table 1:** Number of differentially expressed genes in different sample types regarding the general,
157 population-specific, phenotype-specific, and line-specific response to cold acclimation

	total expressed genes	up regulated	down regulated	total DE
General response to acclimation				
Whole body	14304	3825 (27%)	3930 (27%)	7755 (54%)
Malpighian tubule	12189	3280 (27%)	2874 (24%)	6154 (50%)
Hindgut	13272	3715 (28%)	3480 (26%)	7195 (54%)
Population-specific response				
Whole body				
BKK	14300	3618 (25%)	3668 (26%)	7286 (51%)
KATH	14300	3414 (24%)	3497 (24%)	6911 (48%)
KATH vs BKK*	13191	1181 (9%)	827 (6.3%)	2008 (15%)
Malpighian tubule				
BKK	11721	3077 (26%)	2606 (22%)	5683 (48%)
KATH	11191	2288 (20%)	2174 (19%)	4462 (40%)
KATH vs BKK*	9125	124 (1.4%)	92 (1%)	216 (2.4%)
Phenotype-specific response				
Hindgut				
Fast	12821	3203 (25%)	3570 (28%)	6773 (53%)
Slow	12547	2907 (23%)	2784 (22%)	5691 (45%)
Fast vs Slow*	12547	182 (1.5%)	246 (2%)	428 (3.4)
Line-specific response**				
Whole body				
FastBKK	14300	3333 (23%)	3434 (24%)	6767 (47%)
SlowBKK	14300	3091 (22%)	3300 (23%)	6391 (45%)
FastBKK vs SlowBKK*	13468	508 (3.8%)	472 (3.5%)	980 .3%)

158 * For these comparisons, the columns show the number of genes that are significantly differentially
159 expressed between the compared groups. Up and down-regulated columns indicate direction regarding the
160 effect of acclimation on the former group compared to the latter group.

161 ** Only iso-female lines of the ancestral population are taken into consideration.

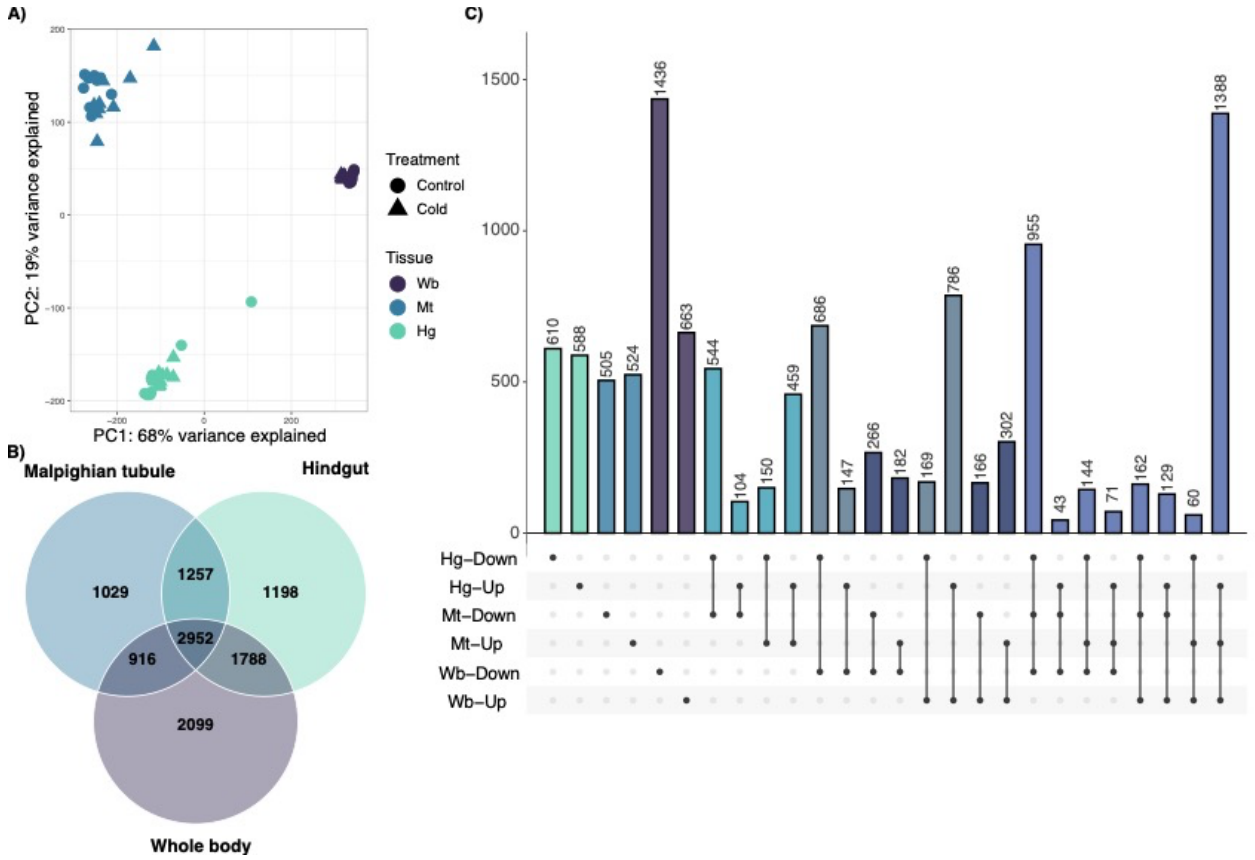

Figure 1: Transcriptomic response to cold acclimation in ionoregulatory tissues and the whole body. A) Principal component analysis. The samples are shown according to the tissue (color) and treatment (shape). Whole body (Wb), Malpighian tubule (Mt), and hindgut (Hg) tissues are represented by purple, blue, and green colors, respectively. The control samples are represented by circles and the cold acclimated samples are represented by triangles. B) Venn diagram showing the number of DE genes specific to or shared among sample types: Whole body, Malpighian tubule and Hindgut samples exhibited both shared, as well as exclusive differentially expressed genes. C) Upset plot showing differentially expressed genes and their overlap between the three different sample types. Black, horizontal bars indicate the total number of differentially expressed genes that were significantly up- or down-regulated in a sample in response to cold acclimation. A black circle indicates that a sample and direction (up/down) combination is included in an intersection class. Intersection classes with a single circle are comprised of genes significantly differentially expressed in a single tissue and direction. Circles connected by a line indicate intersection classes comprised of multiple tissue and direction combinations. Vertical bars correspond to the number of genes in each intersection class, colors represent the respective sections of the Venn diagram.

Strong systemic transcriptional response to cold acclimation

We performed RNA sequencing on whole-body samples to obtain a systemic overview of the transcriptional response to cold acclimation in *Drosophila ananassae*. This approach has the unique advantage of being both cost-efficient and unbiased in terms of preconditions on

181 transcription. Samples of acclimation and control treatments were clustered according to the
182 populations of origin (Figure 2A). The first principal component explained 58% of the variance.
183 It separated cold-acclimated samples from control samples, while the second principal component
184 explained 14% of the variance and separated the derived population from the ancestral population.
185 Within the ancestral population, the samples also clustered according to their CCRT phenotype
186 (Figure 2B). We addressed three questions with this data: (1) What is the general, systemic
187 transcriptional response to cold acclimation? (2) How does transcriptional response differ between
188 ancestral and derived populations? (3) How does transcriptional response differ between cold-
189 tolerant and cold-sensitive strains in the ancestral population?

190 More than half of the transcriptome (54%) was differentially expressed between control and
191 treatment, with 3825 genes significantly up-regulated and 3930 genes significantly down-
192 regulated (Table 1). Gene ontology enrichment analysis revealed that up-regulated genes were
193 enriched in biological processes related to translation and cell junction organization (Figure 2C,
194 Supplementary Table 5). On the contrary, down-regulated genes were enriched in metabolic
195 processes (Figure 2D, Supplementary Table 5). These results suggest a solid response to cold
196 acclimation in the whole body, and the transcriptional response of populations differs not only in
197 the presence of cold treatment but also at the basal level.

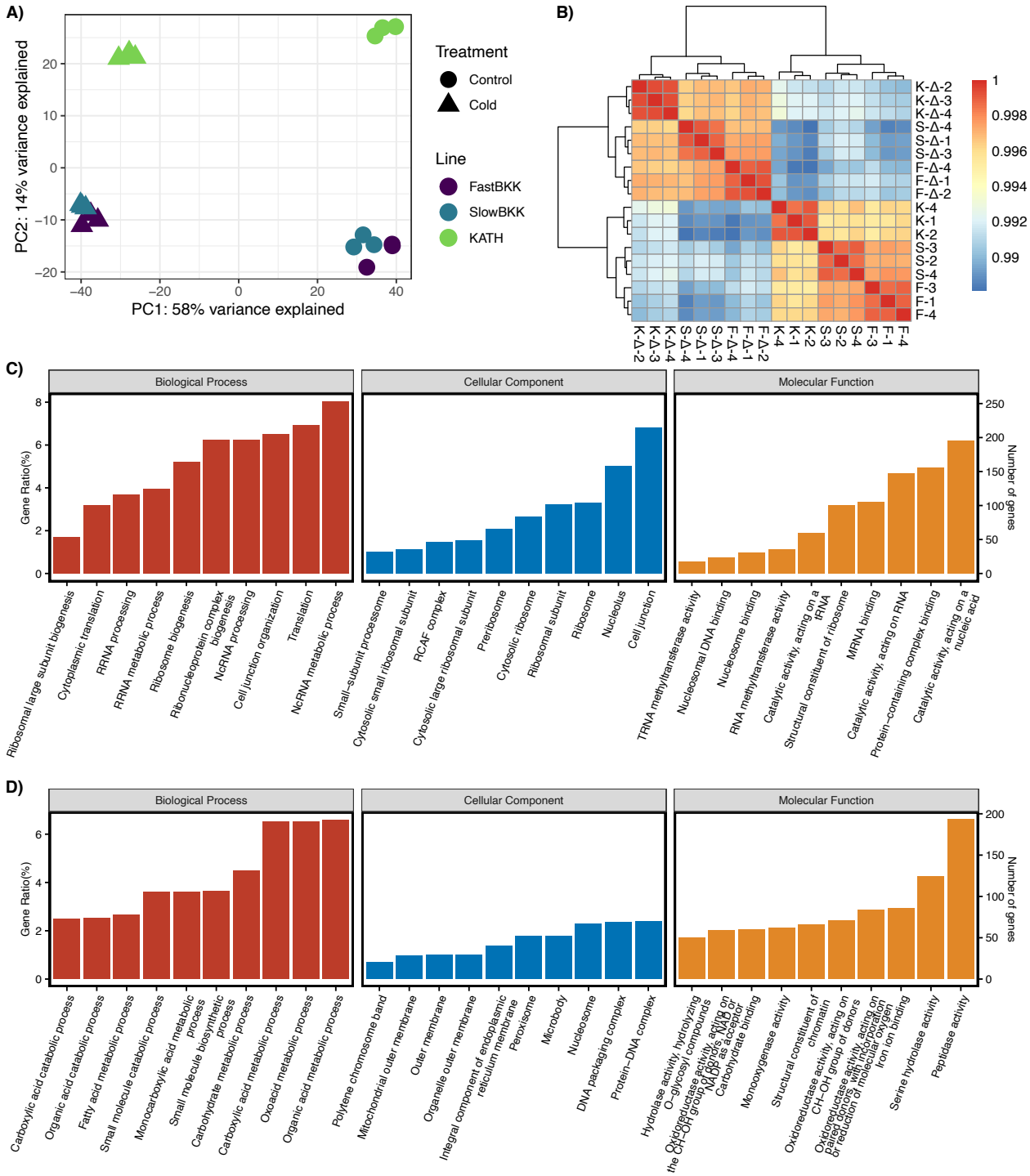

Figure 2: Transcriptomic response to cold acclimation in the whole body. A) Principal component analysis. The samples are shown according to the pooled strains (color) and treatment (shape). FastBKK, SlowBKK, and KATH sample pools are represented by purple, blue, and green colors, respectively. The control samples are represented by circles and the cold acclimated samples are represented by triangles. B) Heatmap for the sample pools. Sample clusters are shown on the left and top of the heatmap. The similarity matrix indicates high (red) and low (blue) similarity of samples. Gene ontology enrichment analysis results for genes C) up-

205 regulated and D) down-regulated in the whole body in response to cold acclimation. Biological processes
206 (red bars), cellular components (blue bars), and molecular functions (yellow bars) are shown in separate
207 panels.

208 **Systemic differences between populations are mediated by genes involved in detoxification**
209 **and maintaining ion balance**

210 To characterize the differences between populations, we contrasted the transcriptional response of
211 ancestral and derived populations. A total of 14300 genes were expressed in each population (Table
212 1). In the ancestral population, 51% of these genes were differentially expressed upon cold
213 acclimation, while 3618 genes were significantly up- and 3668 down-regulated, respectively. In
214 comparison, 48% of these genes were differentially expressed in the derived population, while
215 3414 genes were significantly up-regulated, and 3497 genes were significantly down-regulated.
216 Although the populations shared over half of the differentially expressed genes (Supplementary
217 Figure 2A), 2008 genes showed significant differential expression between populations in
218 response to cold acclimation. The GO analysis suggested that the genes with differential expression
219 between the populations were mainly located in muscle tissues and enriched in cilium movement
220 and various metabolic processes, including fatty acid metabolism (Supplementary Figure 2B).
221 Notably, these genes were also enriched in molecular functions related to ion binding, ion channel,
222 and oxidoreductase activity (Supplementary Table 6). Overall, these results suggest the difference
223 between the acclimation response of populations ground on ion transport and detoxification.

224 **The actin cytoskeleton differentiates the acclimation response of tolerant and sensitive**
225 **strains in the ancestral population**

226 To address the question of how the transcriptional response differs within the ancestral population
227 between cold-tolerant and cold-sensitive strains, we compared gene expression upon cold
228 acclimation in both types of strains. For the cold-tolerant strains, 3333 genes were significantly

229 up-regulated, and 3434 genes were significantly down-regulated (Table 1). For the sensitive
230 strains, 3091 genes were significantly up-regulated, and 3300 genes were significantly down-
231 regulated. However, 980 genes were differentially expressed between cold-sensitive and cold-
232 tolerant strains. GO enrichment analysis revealed significant enrichment of these genes in various
233 biological processes related to the actin cytoskeleton. (Supplementary Table 7)

234 **Adaptive transcriptional response is evident in the renal tissues**

235 Malpighian tubules (Mt) are the renal equivalent of mammals in insects and contribute to
236 maintaining homeostasis, which can be disrupted by cold exposure. Therefore, we investigated the
237 transcriptional response of Mt to characterize the impact of cold acclimation on renal tissues in *D.*
238 *ananassae*. Like the whole-body samples, RNA samples extracted from Mt of acclimated and
239 control flies were clustered according to the populations of origin, suggesting that the populations
240 are distinct in their response to acclimation and at the basal level of gene expression (Figure 3A).
241 Principal component analysis indicated that 37% of the variation can be explained by the first
242 principal component, which separated cold-acclimated and control samples, and 14% of the
243 variation can be explained by the second principal component, which separated the ancestral and
244 derived populations. Therefore, we investigated the general transcriptional response of Mt to cold
245 acclimation and the population-specific response.

246 In the Mt samples, 3280 genes were up-regulated, and 2874 were down-regulated (Table 1).
247 Similar to the whole-body samples, up-regulated genes of Mt were enriched in GO terms related
248 to translation and transcription (Supplementary Figure 3A, Supplementary Table 8), and the down-
249 regulated genes were enriched in processes related to fatty acid degradation (Supplementary Figure
250 3B, Supplementary Table 8). However, contrary to the whole-body samples, the down-regulated

genes of the Mt were also enriched in chromatin remodeling. Additionally, a major part of the down-regulated genes was located in the mitochondrion and, more specifically, the respiratory chain complex.

255 **Figure 3:** Transcriptomic response to cold acclimation in the Malpighian tubules. A) Principal component
256 analysis. The samples are shown according to the pooled strains (color) and treatment (shape). FastBKK,
257 SlowBKK, and KATH sample pools are represented by purple, blue, and green colors, respectively. The
258 control samples are represented by circles and the cold acclimated samples are represented by triangles. B)
259 Venn diagram showing the number of DE genes specific to or shared between populations: Bangkok and
260 Kathmandu populations exhibited both shared, as well as exclusive differentially expressed genes in
261 response to acclimation. Gene ontology (GO) enrichment analysis results for genes C) up-regulated and D)
262 down-regulated in the Malpighian tubules of Kathmandu population compared to Bangkok population in
263 response to cold acclimation. Biological processes (red bars), cellular components (blue bars), and
264 molecular functions (yellow bars) are shown in separate panels.

265 **Differences in renal tissues of populations are mediated by genes involved in ion transport**
266 **and lipid metabolism**

267 In order to understand the differences in metabolic processes involved in cold adaptation between
268 the ancestral and derived populations, we compared the transcriptional response to cold
269 acclimation in flies from the Bangkok and Kathmandu populations. In the ancestral population,
270 11721 genes were expressed, with 3077 up-regulated and 2606 down-regulated (Figure 3B, Table
271 1). In the derived population, 11191 genes were expressed, with 2288 up-regulated and 2174 down-
272 regulated. Among these DE genes, 216 genes showed differential expression between the
273 populations. We conducted a GO analysis to explore the functions of these genes and found GO
274 terms related to transposition. To enhance the analysis, we performed separate analyses for gene
275 sets: genes up regulated in the derived population compared to the ancestral one, and *vice versa*.
276 The analysis showed that genes up regulated in the Kathmandu population compared to Bangkok
277 were enriched in molecular functions related to ion transport (Figure 3C, Supplementary Table 9).
278 Conversely, genes up regulated in the ancestral population compared to the derived one were
279 enriched in processes and functions related to transposition (Figure 3D, Supplementary Table 9).
280 Additionally, this analysis revealed an enrichment in molecular functions related to fatty acid ligase
281 activity.

282 **Hindgut responds to cold acclimation with respect to phenotype, but not to population**

283 Along with the Malpighian tubules, hindguts (hg) are essential in regulating ion and water balance.
284 Therefore, we investigated the tissue-specific transcriptional response to cold acclimation in the
285 hindgut of *D. ananassae* flies to elucidate molecular mechanisms of cold adaptation in insects.
286 Initial PCA revealed two clusters and an outlier sample (Figure 4A). When the outlier was removed
287 to increase the resolution, PC1 explained 48% of the variance, and PC2 explained 12%, separating
288 the treatments and phenotypes, respectively (Supplementary Figure 4A). The acclimated samples
289 clustered according to their cold tolerance phenotype, in other words, according to their recovery
290 time from chill coma. However, as opposed to the whole body and MT, the control samples showed
291 no apparent separation, which suggests that the strains do not differ at their basal gene expression
292 level according to their cold tolerance phenotype or population of origin. Therefore, we addressed
293 two questions with this data: (1) What is the general response to cold acclimation in the hindguts?
294 (2) How does transcriptional response differ between cold-tolerant and cold-sensitive strains?

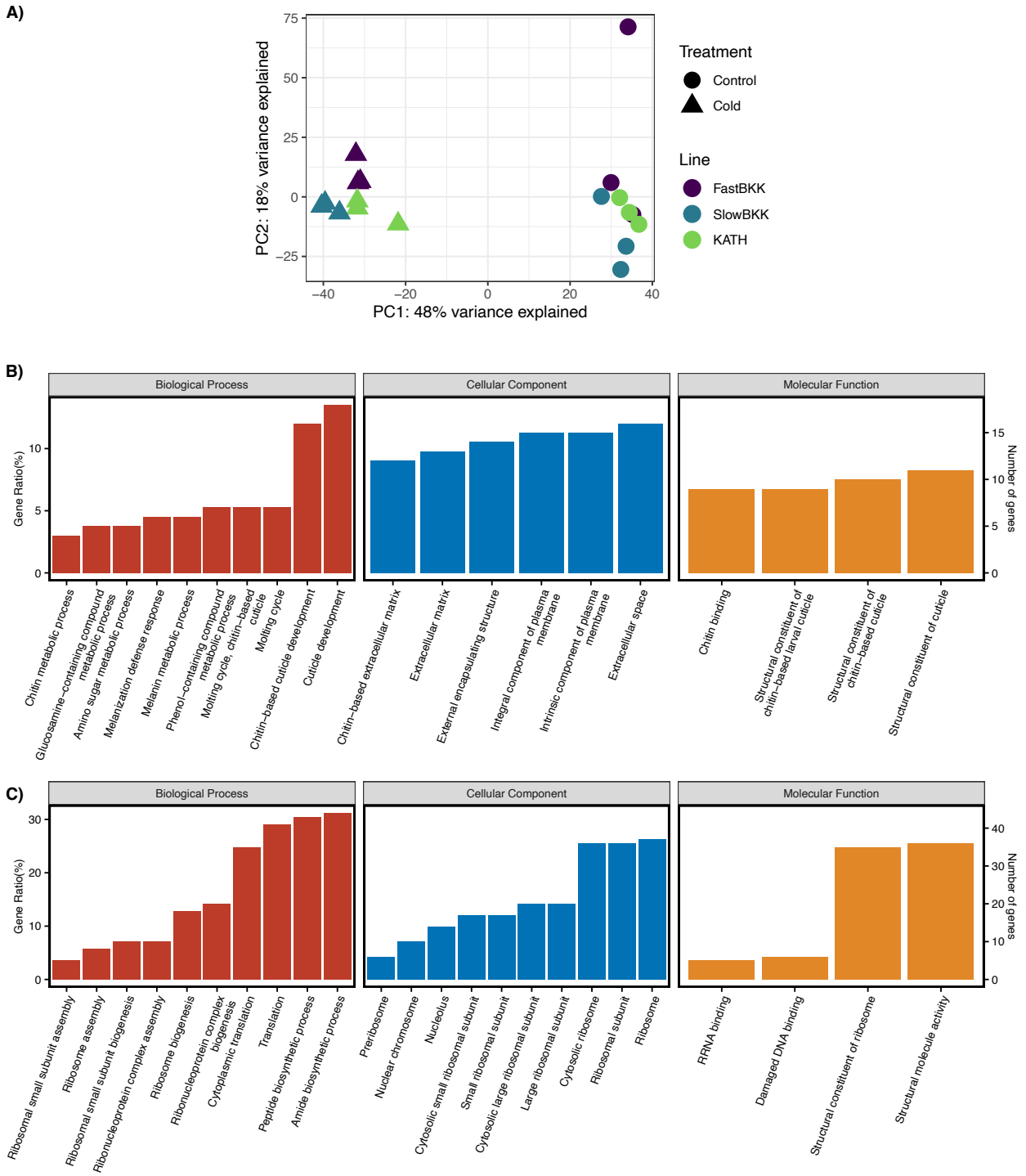

Figure 4: Transcriptomic response to cold acclimation in the hindguts. A) Principal component analysis. The samples are shown according to the pooled strains (color) and treatment (shape). FastBKK, SlowBKK, and KATH sample pools are represented by purple, blue, and green colors, respectively. The control samples are represented by circles and the cold acclimated samples are represented by triangles. Gene ontology (GO) enrichment analysis results for genes B) up-regulated and C) down-regulated in the hindguts of tolerant flies (fast phenotype) compared to sensitive flies (slow phenotype) in response to cold acclimation.

302 Biological processes (red bars), cellular components (blue bars), and molecular functions (yellow bars) are
303 shown in separate panels.

304 In the hindgut samples, we observed a significant up-regulation of 3835 genes and a down-
305 regulation of 3483 genes in response to cold acclimation (Table 1). Like the whole body and
306 Malpighian tubules, the up-regulated genes in the hindguts were enriched in GO terms related to
307 translation and transcription (Supplementary Figure 4B, Supplementary Table 10). In contrast, the
308 down-regulated genes showed enrichment in biological processes related to cellular respiration
309 and lipid metabolism, with many of these genes located in the mitochondrial membrane
310 (Supplementary Figure 4C, Supplementary Table 10).

311 **Cellular respiration and chitin metabolism play roles in the acclimation response of tolerant**
312 **and sensitive strains in the hindgut**

313 We characterized the transcriptional response to cold acclimation in cold-tolerant and cold-
314 sensitive strains with differential gene expression analysis. In the cold-sensitive strains, 3046 genes
315 were significantly up-regulated, and 2886 genes were significantly down-regulated in response to
316 acclimation (Table 1). On the other hand, in the cold-tolerant strains, 3401 genes were significantly
317 up-regulated, and 3224 genes were significantly down-regulated in response to cold acclimation.
318 Between the tolerant and sensitive strains, 330 genes were differentially expressed. GO enrichment
319 analysis revealed that these genes were enriched in biological processes related to translation and
320 ribosome biogenesis and in molecular functions related to chitin binding. We separately
321 investigated the genes up-regulated in the cold-tolerant strains compared to the sensitive strains
322 and *vice versa*. Genes significantly up-regulated in the cold-tolerant strains compared to the cold-
323 sensitive strains were enriched in GO terms related to cuticle development, respiration, and
324 metabolism (Figure 4B, Supplementary Table 11). Additionally, the products of these genes were
325 localized mainly to the membrane and extracellular matrix. Genes significantly up-regulated in the

326 cold-sensitive strains compared to the cold-tolerant strains were enriched in GO terms related to
327 translation (Figure 4C, Supplementary Table 11).

328 **The network analysis provides an overview of the cold acclimation response**

329 In addition to differential expression and gene ontology analyses, we conducted a weighted gene
330 co-expression network analysis (WGCNA) to uncover the pathway networks crucial to the cold
331 acclimation response in the whole body, Malpighian tubules, and hindguts of *D. ananassae*. The
332 general response to acclimation in the whole body was found to be centered around signaling
333 pathways and nervous system development (Supplementary Figure 5A), as per WGCNA, while
334 the populations showed variations in lipid and amino acid metabolism (Supplementary Figure 5B-
335 C). In the Malpighian tubules, the WGCNA revealed pathways involved in RNA metabolism and
336 WNT signaling (Supplementary Figure 5D-E). Conversely, the analysis of hindgut samples
337 unveiled carbohydrate metabolism-related pathways (Supplementary Figure 5F). Notably, no
338 population- or phenotype-specific differences were observed in the ionoregulatory tissues.

339 **Distinct transcriptional response to cold acclimation and cold shock**

340 To receive a comprehensive overview of cold tolerance measured by distinct phenotypes, we
341 contrasted our previous results of the transcriptional response to cold shock as measured by chill
342 coma recovery time upon cold shock (Königer & Grath, 2018) with the transcriptome data from
343 this study. To this end, we considered the genes differentially expressed in the cold-tolerant and
344 cold-sensitive strains of the ancestral population from Bangkok, Thailand, in two conditions: (1)
345 cold shock and (2) cold acclimation.

346 Our findings reveal the intricate nature of gene expression in response to cold shock and cold
347 acclimation. We observed that more genes are differentially expressed in response to cold

348 acclimation than cold shock (Supplementary Figure 6), indicating a more complex regulatory
349 network in the former. To understand these responses, we investigated the up- and down-regulated
350 genes in both treatments and performed a GO enrichment analysis.

351 The genes up-regulated in response to both treatments were enriched in actin cytoskeleton-related
352 GO terms (Supplementary Table 12). In contrast, the down-regulated ones showed enrichment in
353 various metabolic processes such as organic acid, lipid, and carbohydrate metabolic processes
354 (Supplementary Table 12). We also investigated the genes with opposite expression (i.e., up-
355 regulated in one treatment but down-regulated in the other) in response to treatments. Among the
356 1932 genes that showed differential expression in both studies, 390 genes had opposite directions.
357 The genes up-regulated upon cold shock but down-regulated upon acclimation were enriched in
358 the immune response (Supplementary Table 13). On the other hand, the genes that were up-
359 regulated in response to acclimation but down-regulated after cold shock showed enrichment in
360 biological processes related to transcription and translation (Supplementary Table 13).

361 Finally, we investigated the genes that show differential expression in response to only one of the
362 treatments. Over 5000 genes were up- or down-regulated upon acclimation, but only 1070 genes
363 were uniquely expressed after cold shock. The genes differentially expressed only upon the cold
364 shock treatment were enriched in protein folding, actin cytoskeleton organization, replication, and
365 glycosylation (Supplementary Table 14). The genes that were differentially expressed upon
366 acclimation showed enrichment in translation and transcription as well as amino acid metabolism
367 and copulation (Supplementary Table 14).

Discussion

Understanding the differences between the ancestral and derived populations and the variance within the ancestral Bangkok population sheds light on the adaptive processes leading to increased cold tolerance of the species. The two populations were tested for their acclimation response. Both populations showed improved cold tolerance upon acclimating to mild temperatures as expected, but the underlying mechanisms were unknown. Comparative transcriptomics using the whole-body samples provided the systemic overview to understand the differences in the acclimation response between the two populations and within the ancestral population. Additionally, the tissue-specific response to acclimation provided further insight into population and phenotypic differences.

In a complex phenotype, such as cold response, transcriptomic analysis of a whole body might lead to missing information (reviewed in Hoedjes *et al.*, 2024). However, tissue-specific RNA-Seq can unmask details otherwise lost in the systemic approach. Both whole-body and tissue-specific approaches were used in this study to increase the strength of the comparison of populations, phenotypes, and strains. The DE genes in the Malpighian tubule samples enriched in hypoxia response and HSP binding activity are not differentially expressed in the whole-body samples. Similarly, the DE genes in the hindgut samples enriched in sodium channel activity and cuticle structure are not differentially expressed in the whole body. On the other hand, even though the genes enriched in cellular respiration were up-regulated in the whole body, they were down-regulated in both ionoregulatory tissues. Taken together, combining whole-body and tissue-specific RNA-Seq allowed combining advantages while eliminating the disadvantages of each approach.

390 Previous studies on the cold acclimation response of other *Drosophila* species suggest that the
391 percentage of differentially expressed genes in response to acclimation decreases as the species
392 becomes more tolerant to cold (Parker *et al.*, 2015, 2021; MacMillan *et al.*, 2016; Enriquez &
393 Colinet, 2019). Even though these differences can be due to technological advancements,
394 acclimation conditions, or analysis methods, the current study's large-scale differential expression
395 aligns with this claim. In addition, the general response to cold acclimation consisted of differential
396 expression of genes related to translation, transcription, and metabolism. Over-expression of
397 translation-related genes parallels the high number of DE genes responding to acclimation. The
398 populations also showed a slight difference in DE gene percentage, supporting the previous
399 suggestion.

400 Exposure to low temperatures decreases membrane fluidity (Steponkus, 1984; Drobnis et al., 1993;
401 Koštál, 2010). Homeoviscious adaptation suggests that the organism can adjust its membrane
402 fluidity by increasing the proportion of unsaturated fatty acids or the ratio of short-chain fatty acids
403 to long-chain fatty acids (Sinensky, 1974; Koštál, 2010). Insects can adjust their membrane
404 composition through fatty acid metabolism to keep the membrane fluidity consistent, which may
405 increase cold tolerance during acclimation (reviewed in Teets & Denlinger, 2013). The fatty acid
406 elongation was down-regulated in response to acclimation in both populations, preventing a
407 decrease in the short-chain-to-long-chain ratio. However, the differential expression analysis
408 between the populations suggested that the down-regulation of genes related to fatty acid elongase
409 activity was significantly higher in the Bangkok population than in the Kathmandu population.
410 These results suggest that the ancestral population prevents the elongation of short-chain fatty
411 acids into long-chain ones to maintain the membrane fluidity. In contrast, the acclimation response
412 of the derived population does not solely depend on membrane structure.

As discussed previously, cold exposure increases potassium ion concentration in the extracellular space, resulting in membrane depolarization (Hoyle, 1953; Findsen *et al.*, 2014; reviewed in Overgaard & MacMillan, 2017 and in Overgaard *et. al*., 2021). As acclimation increases tolerance, adjustments are expected to be made to improve the maintenance of the water-ion balance. Hence, the up-regulation of genes regulating ion transport mechanisms can improve the cold tolerance phenotype. The genes significantly up-regulated in the derived population compared to the ancestral one showed enrichment in ion transport and channel activity. Specifically, the up-regulation of genes regulating the potassium channel activity in the Kathmandu population indicates that the derived population takes advantage of preventing depolarization by maintaining the potassium concentration. On the other hand, in the Malpighian tubules, the ion transport genes were responsible for the active transmembrane transport rather than ion-specific channels. As active transport is affected more than passive diffusion in the case of cold exposure (Koštál *et al.*, 2004; Zachariassen *et al.*, 2004; MacMillan & Sinclair, 2011), maintaining the ion balance in response to acclimation depends on regulating the active transport mechanism.

Taken together, these results suggest that the ancestral population maintains the membrane fluidity to prevent the loss of water-ion balance, whereas the derived population can maintain the active transport in the Malpighian tubules and regulate the potassium ion concentration in the extracellular space in response to acclimation, improving the cold tolerance. Therefore, maintaining ion transport plays a crucial role in the adaptation of the derived Kathmandu population to cold temperatures.

Considering the phenotypic variance within and between populations, comparative transcriptomic analysis of the cold acclimation response also sheds light on the differences between the phenotypes. The analysis focused on the hindgut tissue, where cellular respiration and lipid

436 metabolism-related genes were down-regulated in response to acclimation, whereas cuticle
437 development was up-regulated.

438 A previous study showed that prolonged exposure to cold reduces insect respiration rate (Colinet
439 *et al.*, 2017). However, the same study also suggested that high tolerance can be achieved by
440 maintaining the energy requirement under cold stress. In line with these suggestions, even though
441 the respiration-related genes were down-regulated in response to acclimation in both phenotypes,
442 the difference between the tolerant and sensitive phenotypes was significant: sensitive lines
443 showed significant down-regulation of these genes compared to the tolerant lines. These
444 indications suggest that maintaining the respiration-related processes can be associated with fast
445 recovery from chill coma.

446 Together with respiration, lipid metabolism-related genes were significantly down-regulated in
447 hindguts in response to cold acclimation. As discussed above, low temperatures affect the lipid
448 bilayer structure of the membrane, and these impacts can be overcome by homeoviscious
449 adaptation. Even though the genes associated with fatty acid elongase activity were down-
450 regulated, no phenotype-specific difference was observed.

451 Previous studies showed that reabsorption in the hindgut plays a crucial role in cold tolerance of
452 *Drosophila* species (Andersen *et al*., 2017; Andersen & Overgaard, 2020). Therefore, any
453 adaptation preventing or minimizing the leak in the hindgut is expected to increase tolerance to
454 cold. As the cuticle structure of the hindgut prevents water loss (Phillips, 1970), activation of
455 related pathways is expected to be observed in cold-tolerant strains. Interestingly, genes related to
456 cuticle development and chitin metabolism were up-regulated in the hindguts of all strains in
457 response to cold acclimation. However, the up-regulation significantly differed between the

458 phenotypes. As expected, tolerant strains showed more up-regulation of related genes than the
459 sensitive strains, suggesting that cuticle formation plays a vital role in the cold tolerance level of
460 the species.

461 The comparative transcriptomic analysis of the whole-body cold acclimation response also
462 explains the variation within the ancestral population. Differences were observed in GO terms
463 related to actin cytoskeleton and calcium channels between the fast and slow-recovering ancestral
464 strains. Genes related to both were up-regulated in response to acclimation, but the upregulation
465 was significantly more in the tolerant fast-recovering strains.

466 Actin cytoskeleton plays roles in various biological processes, including cell structure, shape,
467 migration, and signaling. Previous studies suggested that the actin cytoskeleton is essential in cold
468 tolerance (Cottam *et al*., 2006; Kim *et al*., 2006; von Heckel *et al*., 2016; Chen *et al*., 2017; Des
469 Marteaux *et al*., 2018; Bowman *et al*., 2018). One of these studies also showed that the tolerant
470 and sensitive Bangkok strains differed in actin polymerization: six genes had differential
471 expression in response to cold shock (Königer & Grath, 2018). On the other hand, five out of six
472 genes were up-regulated in both phenotypes in response to acclimation. However, they did not
473 show phenotype-specific differential expression even though the initial expression levels were
474 significantly lower in the sensitive phenotype.

475 In addition to the roles above, the actin cytoskeleton also acts as an anchor to ion channels and
476 regulates ion transport (reviewed in Denker & Barber, 2002). Previous studies showed that together
477 with microtubules, actin cytoskeleton dynamics affect the calcium ion influx. Calcium ion
478 concentration is shown to be affected by cold temperatures (Salkoff & Wyman, 1983; Findsen *et*
479 *al*., 2016) and plays an essential role in the activation of apoptotic and necrotic pathways (Koštál

480 et al., 2004, 2007, 2016; Zachariassen et al., 2004; MacMillan et al., 2015, 2016). Genes related
481 to calcium channels were also up-regulated in response to acclimation in the ancestral population.
482 However, the differential expression analysis between the two phenotypes of the ancestral
483 population showed that the calcium transport-related genes were significantly more up-regulated
484 in the fast-recovering phenotype than in the slow-recovering one. These results suggest that the
485 acclimation response of the two phenotypically distinct ancestral pools differ in actin cytoskeleton
486 and ion transport, focusing on calcium ions.

487 In this study, we showed that acclimation to mild temperatures profoundly affected the cold
488 tolerance of *D. ananassae* and investigated the mechanisms leading to increased tolerance. We
489 showed that different populations had distinct transcriptomic responses to acclimation in the whole
490 body and the Malpighian tubules. The differences between populations manifested through ion
491 transport and lipid metabolism. In addition, the cold tolerance phenotype affected the acclimation
492 response in the hindgut: tolerant strains could better maintain the cellular respiration that may
493 generate the energy required by the active transport mechanism and took advantage of the cuticle
494 structure that prevents water loss. Finally, we showed that the phenotypic variation within the
495 ancestral population resulted in differences in the acclimation response, including actin
496 cytoskeleton and calcium channel-related processes.

497 Further studies should be performed to validate these differences between the two phenotypes,
498 focusing on the function of the hindgut and cellular respiration. Hindgut reabsorption functional
499 assays were previously used in larger insects such as grasshoppers (Gerber *et al.*, 2021) but also
500 successfully performed in other *Drosophila* species (Andersen & Overgaard, 2020; Andersen *et*
501 *al.*, 2017). Similarly, cellular respiration measurements were successful in other species (Colinet
502 *et al.*, 2017) and can be used in *D. ananassae*. In addition to functional assays, candidate genes

503 arising from further analyses can be edited through CRISPR-*Cas9* technology previously
504 established in the species (Yılmaz *et al*., 2023) for functional validation.

505 # Materials and Methods

506 **Fly strains**

507 This study used eight *D. ananassae* isofemale lines from Thailand (Bangkok, BKK) and three
508 isofemale lines from Nepal (Kathmandu, KATH). The lines from Bangkok were the same as used
509 in previous studies (Königer and Grath, 2018; Königer *et al.*, 2019). All lines were collected in
510 2002 (Das *et al*., 2004). The fly strains were maintained under standard laboratory conditions
511 (Rearing temperature – RT: 22˚C ± 1˚C 14:10 hours light:dark cycle) at low density on corn-
512 molasses medium. Experiments were performed on flies that were expanded in large vials for two
513 generations under the same conditions.

514 **Cold Acclimation Assay**

515 Acclimation conditions were determined via preliminary tests (see Supplementary Text,
516 Supplementary Methods). Acclimation vials contained standard food and were kept at 10°C for 24
517 hours prior to the experiment to allow the food to cool down to acclimation temperature. Male flies
518 were pooled per population and phenotype on the day of emergence. After 24 hours, flies were
519 transferred to vials containing standard medium as described above. The vials were kept at 10°C
520 for 4 days, followed by 90 minutes recovery time at rearing temperature.

521 **RNA Extraction and Sequencing**

522 Ten individual males per sample were snap-frozen in liquid nitrogen for whole-body samples, and
523 RNA was extracted using MasterPure™ RNA Purification Kit (Epicentre, Madison, WI, USA)
524 following the manufacturer's protocol. For hindgut and Malpighian tubule samples, tissues from
525 60 males per sample were dissected, and the tissues were collected into 1.5 mL Eppendorf tubes
526 containing 10-20 µl 1X PBS. RNA extraction from tissues was performed using RNeasy Mini Kit

527 (Qiagen). RNA quantity and quality for all samples were confirmed with NanoDrop© (ND 1000,
528 VWR International, Radnor, PA, USA) and a bioanalyzer (Bioanalyzer 2001, Agilent
529 Technologies, Santa Clara, CA, USA, provided by the LMU genomics service unit). All samples
530 were sent for sequencing to an external facility (Novogene, Cambridge, UK), which performed
531 poly-A mRNA purification, reverse transcription, and high-throughput using a Novaseq 6000
532 sequencer (Illumina, San Diego, CA, USA) to generate 150-bp paired-end reads. On average, 88
533 million reads were generated per sample. An average of 73 million reads (83%) were successfully
534 mapped to the *D. ananassae* transcriptome (dana_r1.05_FB2016_01) using NextGenMap (version
535 0.5.2) (Sedlazeck *et al.*, 2013) and the annotation of FlyBase release 1.05. This release was used
536 to compare the results of the present study with previously described data on transcriptome
537 response following cold shock (Königer and Grath, 2018). All subsequent analyses were
538 performed in R (version 4.2.2) (R Core Team, 2022).

539 **Differential Gene Expression Analysis**

540 Differentially expressed genes were called using DESeq2 (version 1.38.3) (Love *et al.*, 2014). The
541 general response to treatment was assessed using ~Treatment as the design formula. The
542 differences between other variables (populations, phenotypes, or strains) were assessed using the
543 design formula ~Variable + Treatment + Variable:Treatment. Significant log2 fold changes
544 between control and cold-acclimated samples were determined using a Wald test and a subsequent
545 p-value multiple testing correction according to Benjamini-Hochberg. Genes with a corrected p-
546 value less than 0.05 were considered as significantly differentially expressed.

547 **Gene Ontology (GO) Enrichment Analysis**

548 GO analysis was performed using clusterProfiler (version 4.6.2) (Wu *et al.*, 2021) implemented in
549 R to determine the GO terms associated with *D. melanogaster* orthologs of the differentially
550 expressed genes. The p-values were corrected using FDR method with a p-value cut-off of 0.01.
551 The significant GO terms were visualized using the genekitr package (version 1.2.5) (Liu, 2023).

552 **Weighted Gene Co-expression Network Analysis (WGCNA)**

553 The network analysis was performed using the WGCNA package (version 1.72-1) (Langfelder &
554 Horvath, 2008) to determine co-expressed gene modules and their association with cold
555 acclimation response in specific tissues. The functional annotation of the genes in given modules
556 was performed using packages ReactomePA (version 1.42.0) (Yu & He, 2016) and clusterProfiler
557 to determine the enrichment of Reactome and KEGG pathways, respectively. The visualization
558 enrichment results were visualized using the enrichplot package (version 1.18.4) (Yu, 2023).

559 **Comparison with Cold Shock**

560 Previously, Königer and Grath (2018) analyzed differential gene expression before, at 15 minutes,
561 and 90 minutes after a cold shock in cold-sensitive and cold-tolerant strains of the Bangkok
562 population. The study used the same Bangkok strains used in the current study, but the samples
563 were not pooled for RNA extraction. Additionally, the data contained an extra time point (15
564 minutes after cold shock), which we did not test in the current study. Therefore, we used mapped
565 cold shock reads that were kindly provided by Königer and Grath, analyzed them with the same
566 DESeq2 model as the cold acclimation reads, and compared the DE genes between two phenotypes
567 of the BKK population upon cold acclimation to the DE genes between the same groups at 90
568 minutes after cold shock.

Data Availability

Gene expression data are available at GEO under the accession number GSE270239. All other data and scripts are available in the article and in its Supplementary Material.

Author Contributions

Conceptualization S.G.; investigation V.M.Y., Z.B.; writing-original draft V.M.Y., S.G.; visualization V.M.Y., S.G.; funding acquisition S.G.

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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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Supplementary Material

Navigating the Cold: Integrative Transcriptome Sequencing Approach Reveals Ionoregulatory and Whole-Body Responses to Cold Acclimation in *Drosophila ananassae*

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Supplementary Text

Chill Coma Recovery Time Improves with Acclimation to Mild Temperatures

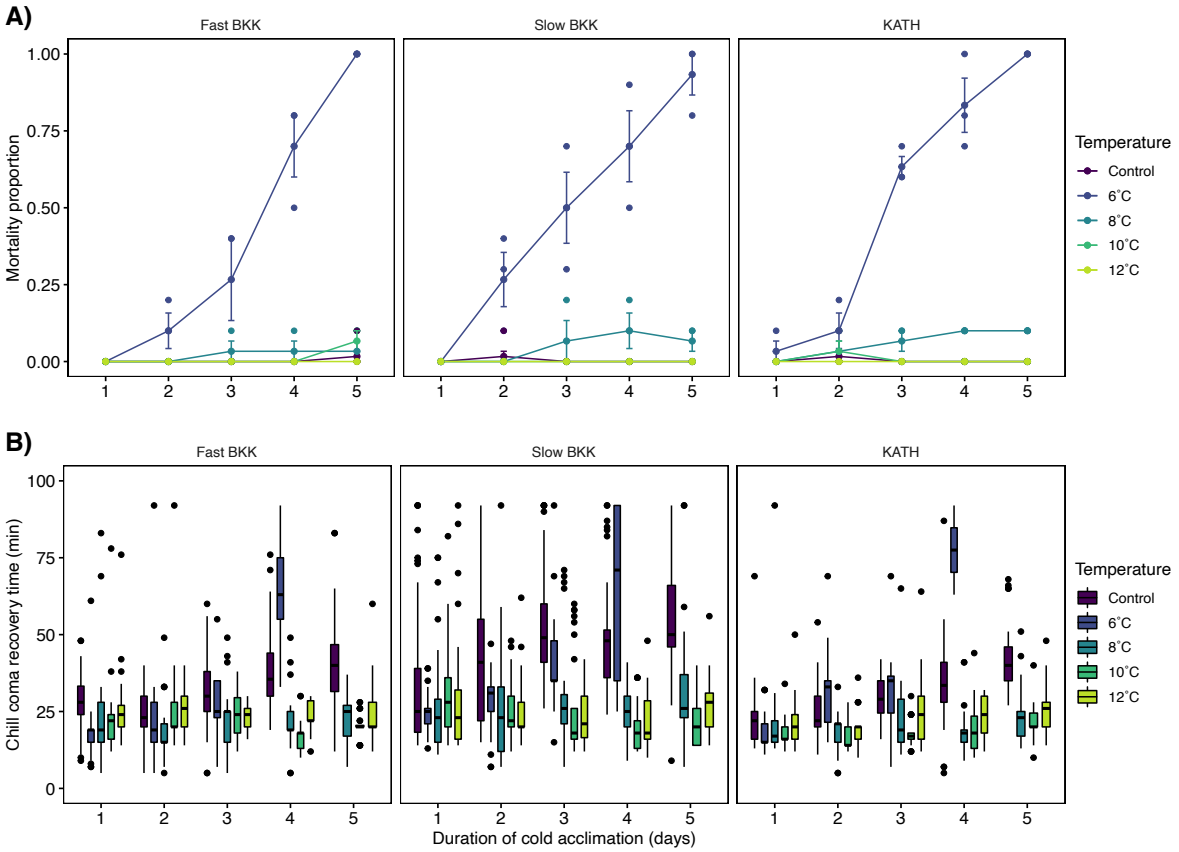
Prior exposure to mild stress conditions can improve the tolerance phenotype. To test the effect of temperature and duration of acclimation on CCRT, we tested four temperatures (6-12˚C) for different periods (1-5 days). We used three strains representing fast-recovering Bangkok (BKK12 for FastBKK), slow-recovering Bangkok (BKK13 for SlowBKK), and Kathmandu (KATH14 for KATH) populations. Flies exposed to 6˚C for more than a day showed high mortality, exceeding 50% on day three and reaching 100% on day 5 (Supplementary Figure 1A, Supplementary Table 1). Additionally, the surviving flies showed CCRT values higher than the control (Supplementary Figure 1B). The remaining temperatures resulted in minimal mortality, reaching an average of 10% at 8˚C and almost no mortality at 10˚C and 12˚C. These temperatures also lead to an improvement in the CCRT phenotype (Supplementary Figure 1B, Supplementary Table 2). Even though the effect of the remaining temperatures did not significantly differ from one another, the difference to control was largest when the acclimation temperature was 10˚C, and the duration was four days, which was chosen as the acclimation set-up for further experiments.

Supplementary Methods

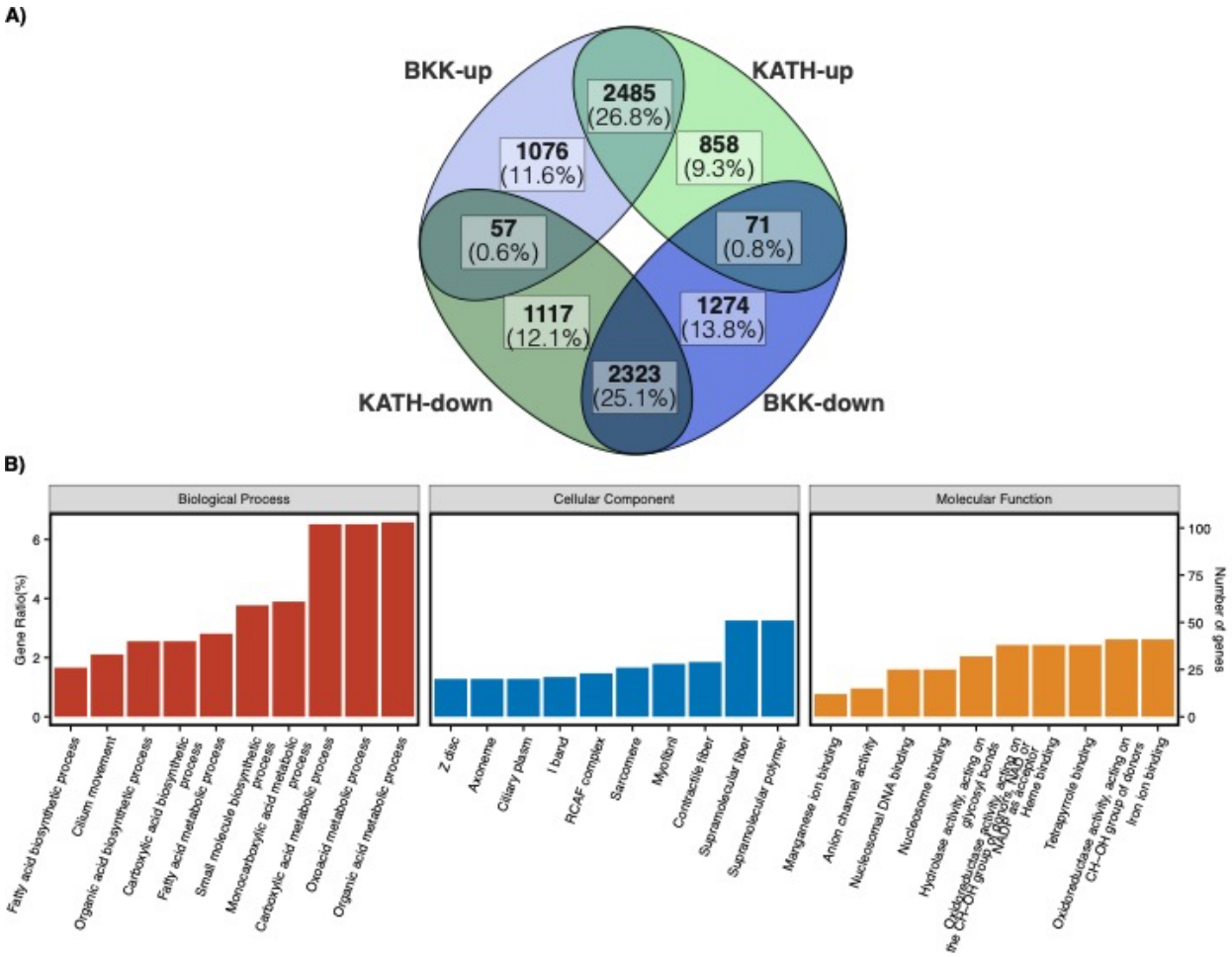
Cold Acclimation Preliminary Assays

To determine the temperature and duration of acclimation, we tested five time periods (1, 2, 3, 4, and 5 days) and four temperatures (6˚C, 8˚C, 10˚C, and 12˚C) using BKK12, BKK13, and KATH14, representing FastBKK, SlowBKK, and KATH populations, respectively. The ranges of temperature and duration were determined based on acclimation studies on other *Drosophila* species. The flies were controlled for age and the effect of acclimation was tested using the survival of acclimated flies and chill coma recovery time (CCRT) upon acclimation. Five replicates were used for each acclimation regime. On the day of emergence, 10 male flies were collected and placed into 50 ml vials with 5 ml food at room temperature. On the following day, test flies were flipped into new vials that were kept at the acclimation temperature and placed into cold incubators for 5-, 4-, 3-, 2-, and 1-day acclimation. The control flies were kept at rearing temperature (22 ± 1°C). After acclimation, the flies were placed back to room temperature for 24 hours for recovery, after which the dead flies were counted and removed from the vials, whereas the surviving flies were flipped into empty vials and placed on melting ice for 3 hours for CCRT measurement. At the end of 3 hours, the vials were placed into room temperature and the recovery time of each fly was measured. The temperature and duration that lead to minimal mortality and improved the CCRT were determined.

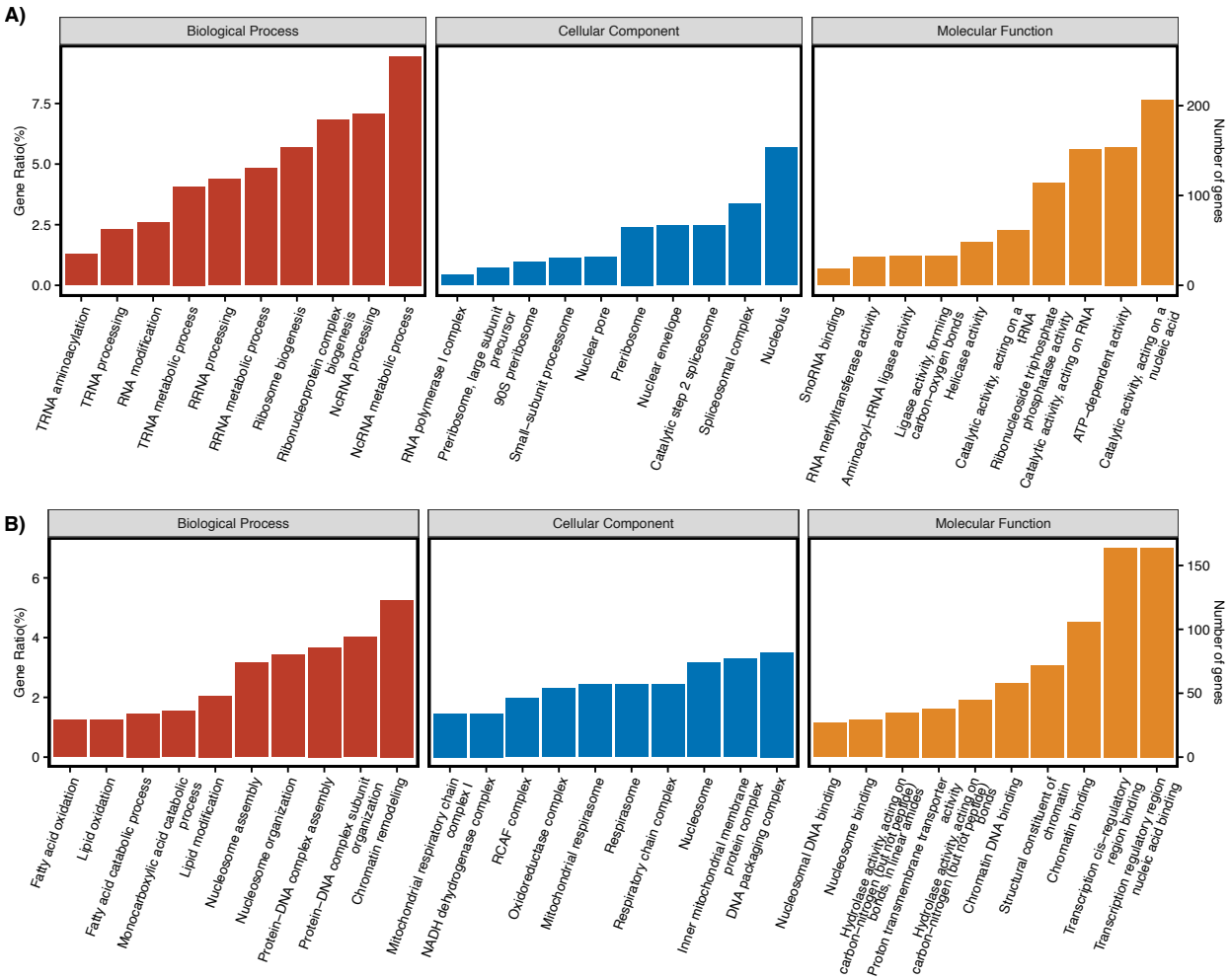
Supplementary Figures



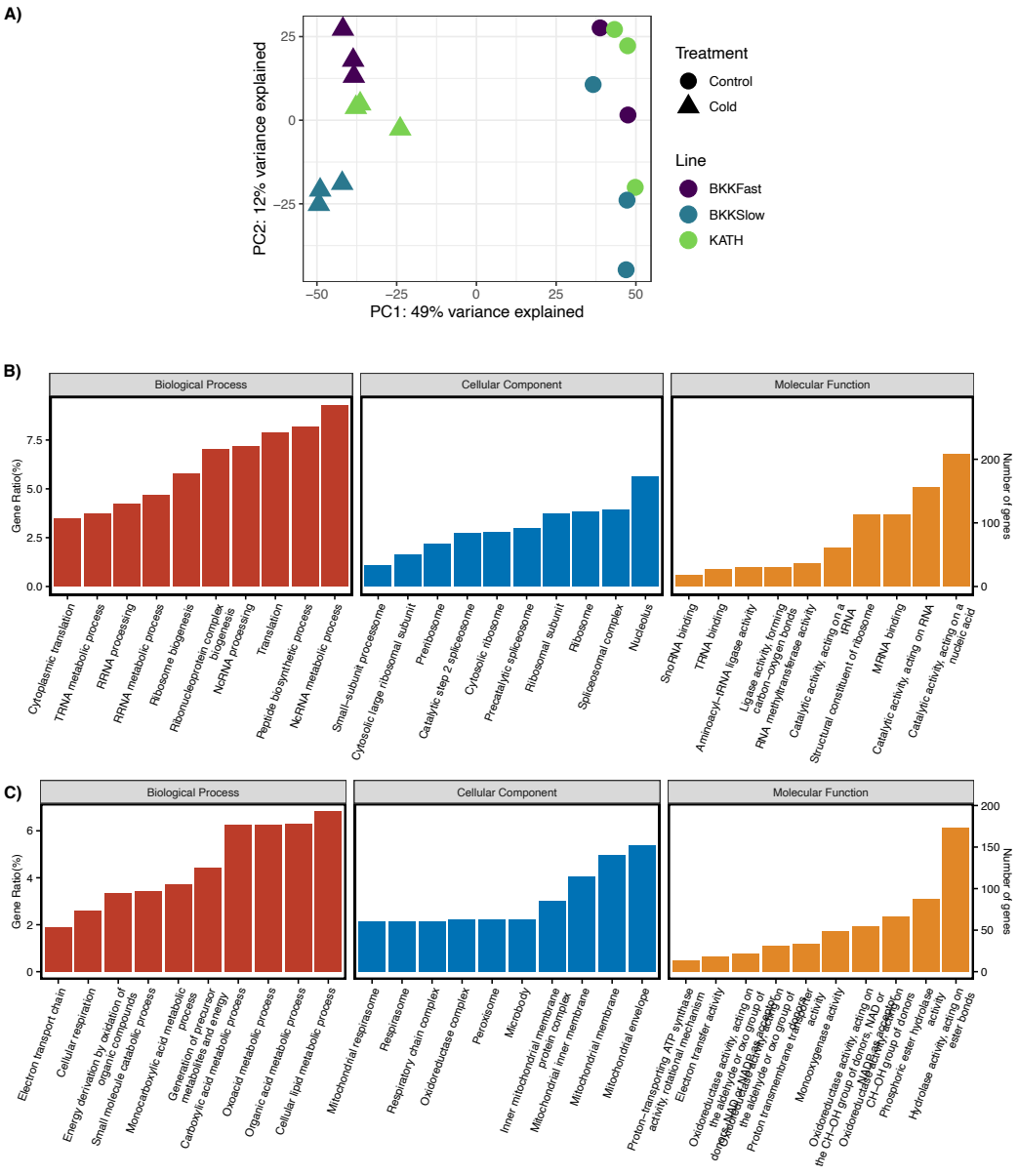
Supplementary Figure 1: Effect of duration of acclimation on A) mortality and B) chill coma recovery time (CCRT) phenotype. The lines that were used were representatives of the respective population and phenotype. Ten flies per replicate in five replicates were used for mortality measurements and dead flies were discarded. The remaining flies were used for chill coma recovery assays. Colored dots indicate proportional mortality of each replicate and black dots indicate outliers with high or low CCRT.



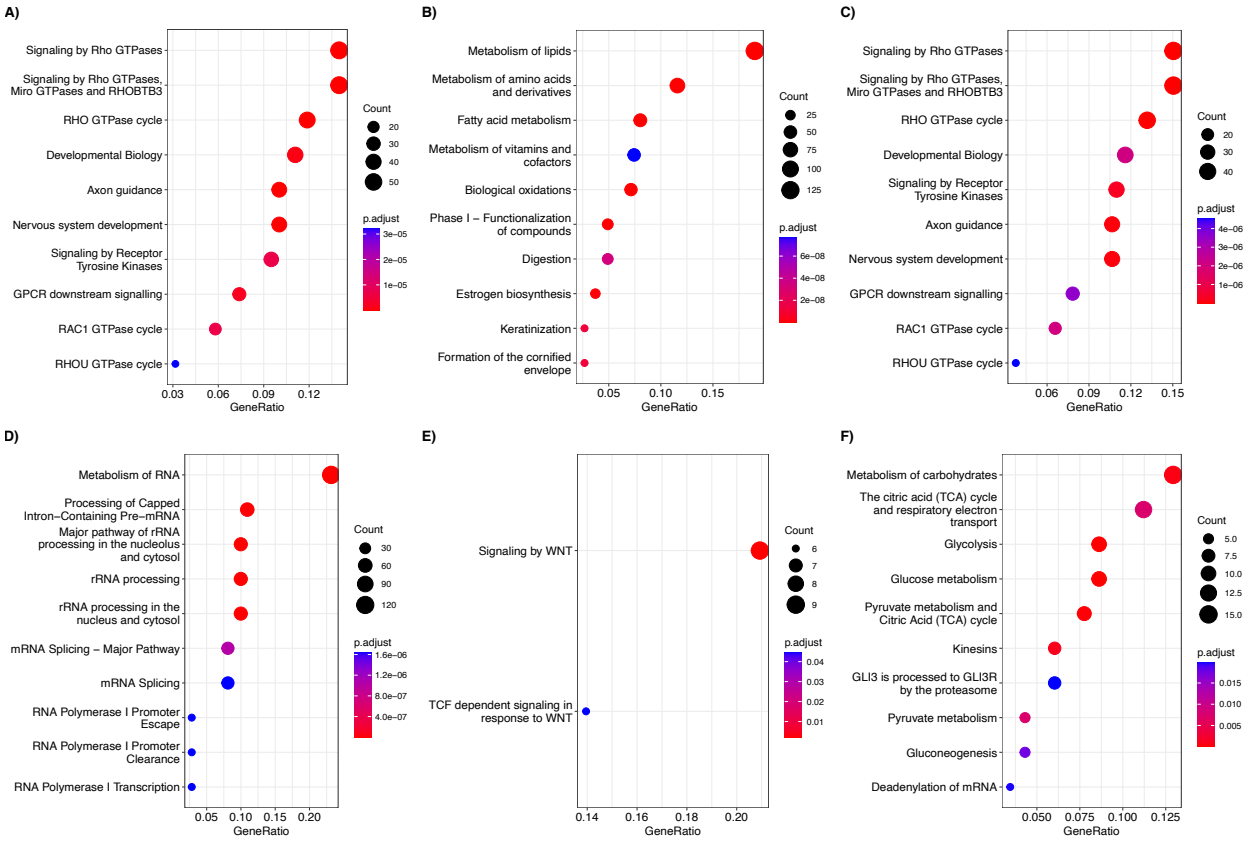
Supplementary Figure 2: A) Venn diagram with the number of differentially expressed (DE) genes specific to or shared between populations. Bangkok and Kathmandu populations exhibited both shared, as well as exclusive differentially expressed genes in response to acclimation. B) Gene ontology (GO) enrichment results for genes differentially expressed between the populations in response to cold acclimation treatment in the whole body. Biological processes (red bars), cellular components (blue bars), and molecular functions (yellow bars) are shown in separate panels.



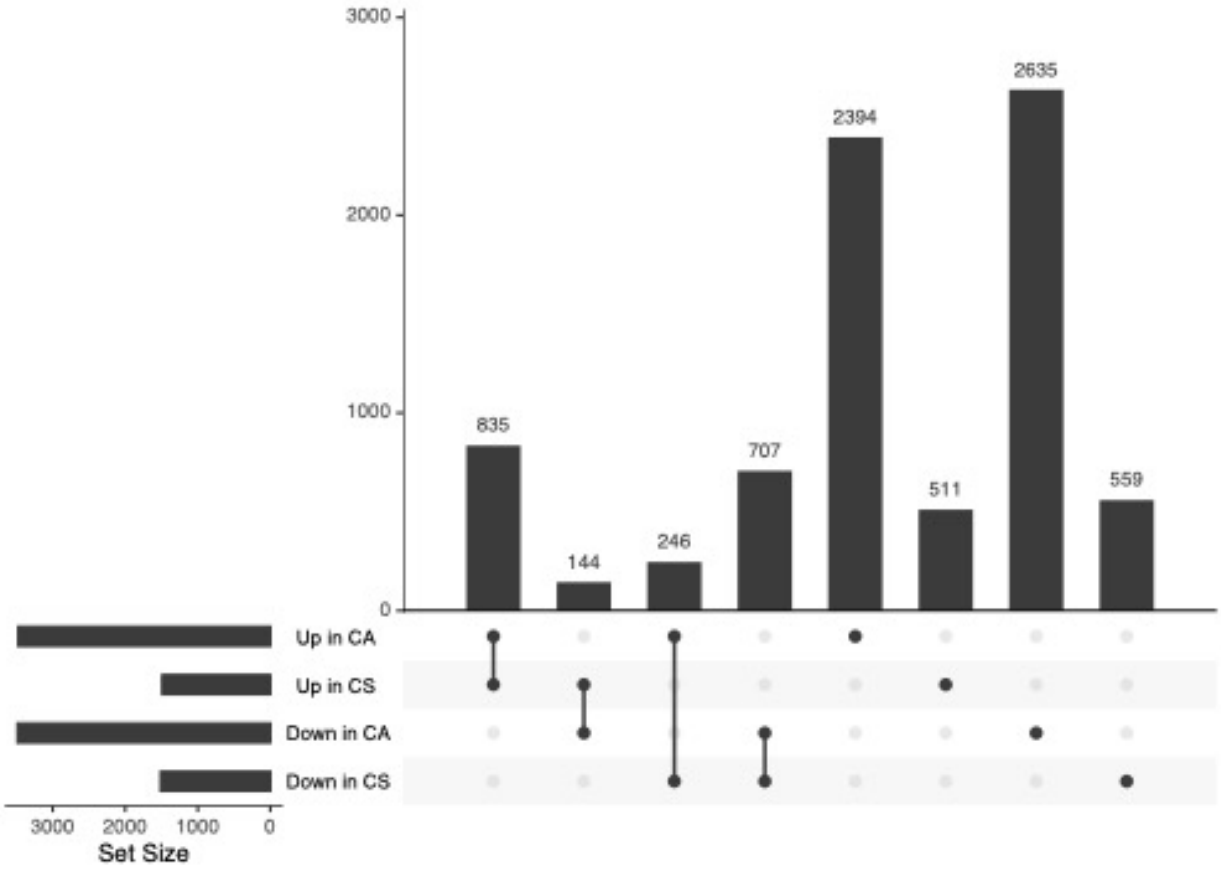
Supplementary Figure 3: Transcriptional response to cold acclimation in the Malpighian tubules. Gene enrichment analysis results for genes A) up-regulated and B) down-regulated in response to cold acclimation. Biological processes (red bars), cellular components (blue bars), and molecular functions (yellow bars) are shown in separate panels.



Supplementary Figure 4: Transcriptional response to cold acclimation in the hindguts. A) Principal component analysis (PCA) after the outlier was removed from the analysis. The samples are shown according to the pooled strains (color) and treatment (shape). FastBKK, SlowBKK, and KATH sample pools are represented by purple, blue, and green colors, respectively. The control samples are represented by circles and the cold acclimated samples are represented by triangles. Gene enrichment analysis results for genes B) up-regulated and C) down-regulated in response to cold acclimation. Biological processes (red bars), cellular components (blue bars), and molecular functions (yellow bars) are shown in separate panels. Cellular respiration and chitin metabolism play roles in the acclimation response of tolerant and sensitive strains in the hindgut.



Supplementary Figure 5: Weighted gene co-expression network analysis (WGCNA). Dot-plots for functional annotation of genes in modules that are significantly enriched in differentially expressed genes in response to cold acclimation in the whole body, Malpighian tubule, and hindgut samples. A) General response to acclimation in the whole body, B-C) population specific response to acclimation in the whole body, D-E) general response to acclimation in the Malpighian tubules, and F) general response to acclimation in the hindguts are shown. The size of the dot represents the gene count associated with the term and the color represents the adjusted p-value.



Supplementary Figure 6: Upset plot showing differentially expressed genes and their overlap between the cold shock (CS) and cold acclimation (CA) assays. Black, horizontal bars indicate the total number of differentially expressed genes that were significantly up- or down-regulated in a sample in response to cold acclimation. A black circle indicates that a sample and direction (up/down) combination is included in an intersection class. Intersection classes with a single circle are comprised of genes significantly differentially expressed in a single treatment and direction. Circles connected by a line indicate intersection classes comprised of multiple treatment and direction combinations. Vertical bars correspond to the number of genes in each intersection class.

Supplementary Tables

Supplementary Table 1: Tukey's HSD test for proportional mortality after cold exposure.

	1-day	2-day	3-day	4-day	5-day
6˚C	e	d	c	b	a
8˚C	e	e	de	de	de
10˚C	e	e	e	e	e
12˚C	e	e	e	e	e
control	e	e	e	e	e

Note 1: Samples that do not share any letter(s) are significantly different. *E.g.* Mortality upon 2-day exposure to 6˚C (**d**) significantly differs from mortality upon 3-day exposure to 6˚C (**c**), but not from mortality upon 3-day exposure to 8˚C (**de**).

Supplementary Table 2: Tukey's HSD test for CCRT values upon acclimation.

	1-day	2-day	3-day	4-day	5-day
8˚C	def	ef	cdef	def	cde
10˚C	def	def	def	f	ef
12˚C	cde	def	def	def	cde
control	cd	c	b	b	a

Note 1: Samples that do not share any letter(s) are significantly different. *E.g.* CCRT of 2-day control samples (**c**) significantly differs from CCRT of 3-day control samples (**b**), but not from CCRT of 1-day control samples (**cd**).
Note 2: Since the flies that survived the acclimation were used in the chill coma recovery assay, 6˚C acclimation was not included in the statistical test for CCRT due to high mortality (50-100%).

Supplementary Table 3: GO enrichment analysis results for differentially expressed genes exclusively in the whole body, Malpighian tubule, or hindgut samples in response to treatment.

Supplementary Table 4: GO enrichment analysis results for differentially expressed genes in the whole body, Malpighian tubule, and hindgut samples in response to treatment in opposite directions.

Supplementary Table 5: GO enrichment analysis results for differentially expressed genes in the whole body in response to treatment.

Supplementary Table 6: GO enrichment analysis results for differentially expressed genes in the whole body in response to treatment. The comparison of Kathmandu and Bangkok populations is shown. The column "Population" indicates which population showed positive log2FC value.

Supplementary Table 7: GO enrichment analysis results for differentially expressed genes in the whole body in response to treatment. The comparison of fast- and slow-recovering Bangkok lines is shown. The column "Line" indicates which line showed positive log2FC value.

Supplementary Table 8: GO enrichment analysis results for differentially expressed genes in the Malpighian tubules in response to treatment.

Supplementary Table 9: GO enrichment analysis results for differentially expressed genes in the Malpighian tubules in response to treatment. The comparison of Kathmandu and Bangkok populations is shown. The column "Population" indicates which population showed positive log2FC value.

Supplementary Table 10: GO enrichment analysis results for differentially expressed genes in the hindguts in response to treatment.

Supplementary Table 11: GO enrichment analysis results for differentially expressed genes in the hindguts in response to treatment. The comparison of fast and slow phenotypes is shown. The column "Phenotype" indicates which phenotype showed positive log2FC value.

Supplementary Table 12: GO enrichment analysis results for differentially expressed genes in the whole body in response to cold acclimation and cold treatment.

Supplementary Table 13: GO enrichment analysis results for differentially expressed genes in the whole body in response to cold acclimation or cold treatment.

Supplementary Table 14: GO enrichment analysis results for differentially expressed genes in the whole body in response to cold acclimation and cold treatment in opposite directions.