The transcriptional basis of cold adaptation in *Drosophila melanogaster*

Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften an der Fakultät für Biologie der Ludwig-Maximilians-Universität München

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Erklärung

Diese Dissertation wurde im Sinne von §12 der Promotionsordnung von Prof. Dr. Wolfgang Stephan betreut. Ich erkläre hiermit, daß die Dissertation nicht einer anderen Prüfungskommission vorgelegt worden ist und daß ich mich nicht anderweitig einer Doktorprüfung unterzogen habe.

Eidesstattliche Versicherung

Ich versichere hiermit an Eides statt, daß die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt worden ist. Im Abschnitt „Contribution and Acknowledgements“ ist mein Anteil an der Arbeit im Einzelnen dargelegt.

München, den 31.01.2017

Korbinian von Heckel

Contents

Contribution and Acknowledgements

All work presented in this thesis has been carried out by me except for the following: The concept of this study was designed by Stephan Hutter, who also performed the mapping of the RNAseq reads. In some of the experiments I had assistance from five Bachelor students that I instructed: Chris Ternes for the plasticity experiments (Chapter 2.2); Veronika Fritsch for the life history trait experiments (Chapter 2.4); Maren Velte, Verena Leingärtner and Theresa Wiebke for the RNAi knockdowns and the subsequent phenotyping (Chapter 2.7). Anne Steincke and Hilde Lainer provided technical assistance in the lab. Stephan Hutter and Wolfgang Stephan gave general guidance throughout the entire course of this thesis.

I am further thankful to Charis Cardeno (UC Davis), Myrto Deligiannaki (LMU Gene Center) and Maria Gambetta (Max Planck Institute for Biochemistry) for providing fly strains and to all members of the LMU Evolutionary Biology Department and my Thesis Advisory Committee for comments and discussion.

This thesis is in large parts based on my earlier publication (see below), which I wrote with input from Stephan Hutter, Wolfgang Stephan, the editor and three reviewers. Many passages of this thesis (in particular in Chapters 2.6, 3.2, 4 & 5) have been adopted verbatim or with minor changes from this publication without explicit citing. Re-used figures and tables, however, are labeled with a citation. Additionally, I refer to some of the large data tables that have been included as supplementary material in this publication.

Original publication

von Heckel K, Stephan W, Hutter S. Canalization of gene expression is a major signature of regulatory cold adaptation in temperate *Drosophila melanogaster*. BMC Genomics. 2016;17:574.

Abbreviations

A157: fly strain from Lake Kariba, Zimbabwe
bp: base pair
CCRT: chill coma recovery time
CCT: chaperonin-containing T-complex
CI: confidence interval
CS: cold shock; for the samples: 3.5h into the cold shock
DEG: differentially expressed gene
DK: fly population from Odder, Denmark
E14: fly strain from Leiden, the Netherlands
GEI: genotype by environment interaction
GO: gene ontology
GWAS: genome-wide association study
Hsp: heat shock protein
IR: inverted repeat
KD: knock down (of gene expression)
L2FC: log2 fold-change
PCA: principal component analysis
qPCR: quantitative real time PCR
QTL: quantitative trait locus
rec15/90: for the samples: 15/90 min into recovery after a 7h cold shock
RG: fly population from Gikongoro, Rwanda
RNAi: RNA interference
RT: room temperature; for the samples: unstressed control
SU: fly population from Umea, Sweden
TPM: transcripts per million
UAS: upstream activating sequence = GAL4 inducible enhancer
VDRC: Vienna Drosophila Resource Center
ZI: fly population from Siavonga, Zambia

Abstract

The overarching goal of this thesis is to identify potential targets and signatures of cold adaptation in *Drosophila melanogaster* with a particular focus on regulatory evolution. The results can roughly be subdivided in three parts:

In the first, I will present cold tolerance related phenotypes in different natural populations and illustrate to what extent they are influenced by environmental variations. Additionally, I will present phenotypic data regarding important life history traits and explore potential fitness trade offs. Two of the studied populations, namely the ones from Sweden and Zambia, exhibit a particularly diverging cold tolerance phenotype, which makes them suitable for the subsequent examination of the genetic basis of this trait difference. While the environment is the major factor influencing cold tolerance, the effects of hereditary adaptation and phenotypic plasticity are additive. Hence, differences between populations persist over a range of environmental conditions. Finally, increased cold tolerance of European strains is not associated with a decrease in reproductive output under standard laboratory conditions.

The second part is based on the transcriptional response towards a cold shock, as measured via qPCR and RNAseq in cold tolerant and cold sensitive populations. Here, I will describe general patterns of the cold shock response and highlight population differences that potentially underlie cold adaptation. Furthermore, I will compare the expression data with a range of similar studies

to identify pervasive characteristics of the transcriptional cold shock response and regulatory adaptation to temperate environments. Irrespective of origin, the transcriptional cold shock response is above all characterized by the swift and massive upregulation of molecular chaperones. Signatures of gene-specific regulatory adaptation regarding the cold shock response are only subtle. However, European populations exhibit a decreased thermosensitivity of gene expression on a genome-wide scale.

The third part is dedicated to functional analyses of candidate genes for cold tolerance. Here, I will report the phenotypic implications of lowered transcript levels for specific candidate genes by employing a genome-wide transgenic RNAi library. Contrary to previous studies, I could not demonstrate a definite phenotypic effect regarding cold tolerance after a single gene knockdown for a range of candidate genes. This further strengthens the conception of cold tolerance as a multigenic trait with presumably minor effect sizes for individual genes.

Zusammenfassung

Das übergeordnete Ziel dieser Studie ist die Identifizierung von potentiellen Angriffspunkten und Charakteristiken der Kälteanpassung in *Drosophila melanogaster* unter besonderer Berücksichtigung regulatorischer Evolution. Die Ergebnisse können grob in drei Teile gegliedert werden:

Im ersten Teil stelle ich die jeweiligen Kältetoleranz-Phänotypen in verschiedenen natürlichen Populationen dar und zeige auf, inwiefern diese von unterschiedlichen Umweltbedingungen beeinflusst werden. Zusätzlich präsentiere ich Daten bezüglich grundlegender Fitness-Parameter und erkunde mögliche Wechselwirkungen zwischen Kälteanpassung und Reproduktionserfolg. Von den untersuchten Populationen zeichnen sich in erster Linie diejenigen aus Schweden und Sambia durch eine stark abweichende Kältetoleranz aus und eignen sich hiermit in besonderem Maße für eine nachfolgende Analyse der genetischen Grundlage dieses Merkmalunterschieds. Die Umwelt ist der Faktor, welcher Kältetoleranz am stärksten beeinflusst, die Auswirkungen von ererbter und erworbener Anpassung sind jedoch additiv. Folglich haben phänotypische Unterschiede zwischen den Populationen über eine große Bandbreite verschiedener Umweltbedingungen Bestand. Die gesteigerte Kältetoleranz europäischer Fliegenlinien geht dabei unter Standardbedingungen nicht mit einem verminderten Fortpflanzungserfolg einher.

Der zweite Teil beruht auf der durch qPCR und RNAseq gemessenen transkriptionellen Reaktion auf einen Kälteschock in kältetoleranten und

kälteempfindlichen Populationen. In diesem Teil beschreibe ich generelle Charakteristika der transkriptionellen Kälteschockreaktion und beleuchte Populationsunterschiede, welche der abweichenden Kälteanpassung zugrunde liegen könnten. Des Weiteren vergleiche ich die Expressionsdaten mit einer Reihe ähnlicher Studien um allgemeingültige Merkmale der Kälteschockreaktion und regulatorischer Anpassung an gemäßigte Klimazonen zu ermitteln. Ungeachtet der Herkunft ist die Kälteschockreaktion vor allem durch die schnelle und umfassende Hochregulierung molekularer Chaperone gekennzeichnet. Genspezifische Anzeichen für regulatorische Anpassung hinsichtlich der Kälteschockreaktion sind lediglich geringfügig ausgeprägt, jedoch weisen europäische Populationen genomweit betrachtet eine verminderte Temperaturempfindlichkeit betreffend ihrer Genexpression auf.

Der dritte Teil behandelt die funktionelle Analyse von Kandidatengenen für Kältetoleranz. Hier untersuche ich die phänotypischen Auswirkungen erniedrigter Transkriptmengen für spezifische Kandidatengene unter Zuhilfenahme einer genomweiten transgenen RNAi-Kollektion. Im Gegensatz zu vorhergehenden Studien konnte ich keine eindeutigen phänotypischen Effekte bezüglich der Kältetoleranz nach einem genspezifischen Knockdown für eine Reihe von Kandidatengenen nachweisen. Dies unterstreicht die Auffassung von Kältetoleranz als einem komplexen Phänotyp mit mutmaßlich geringen Effektgrößen für einzelne Gene.

1. Introduction

In summary, this study constitutes a forward genetic screen. The particular rationale is to use transcriptomic data from cold-tolerant and cold-sensitive populations of *Drosophila melanogaster* at baseline and cold-stressed conditions to identify differences in the cold shock response between the populations and, in the wake thereof, potential targets of cold adaptation.

1.1 The demographic history of *Drosophila melanogaster*

Drosophila melanogaster - nowadays a cosmopolitan human commensal - is of Afrotropical origin and has colonized temperate Eurasian habitats not until after the last glaciation event about 10,000 to 15,000 years ago [Lachaise et al., 1998; Stephan & Li, 2007]. Subsequent spread to the New World has only occurred within the past few hundred years [David & Capy, 1988; Townsend & Rand, 2004; Keller, 2007]. This is supported by the fact that sub-Saharan populations show the most genetic variation [Caracristi & Schlötterer, 2003; Pool et al., 2012] and that polymorphism properties are consistent with population expansion for African populations and with a strong bottleneck for European populations [David & Capy, 1988; Begun & Aquadro, 1993; Glinka *et al.*, 2003]. New World populations are likely the product of admixture between ancestral African and derived European populations [Duchen et al., 2013]. Considering the vast environmental differences between the ancestral species range and newly acquired habitats, local adaptation has been a critical process during this range expansion and has influenced change in

various phenotypic traits [David & Capy, 1988]. Since climate is the major factor influencing insect persistence [Clarke, 1996], the ability to cope with colder temperatures is of prime importance in this regard.

1.2 Chill coma recovery time (CCRT) as a metric for cold tolerance

Chill coma recovery [David et al., 1998] is a widely used and practical laboratory assay to quantify cold tolerance in insects. Below a certain threshold exposition to low temperatures leads to a disruption of osmotic homeostasis resulting in failure of the neuromuscular system and coma [MacMillan et al., 2011; MacMillan et al., 2015]. Restoring the osmotic homeostasis by means of active ion transport is crucial for recovery and metabolically costly [MacMillan et al., 2012]. In *D. melanogaster*, chill coma recovery time (CCRT) has been shown to depend on temperature regimes from the geographical origin of respective strains, in a sense that the colder the climate is in its natural range the faster the strain tends to recover [David et al., 1998; Ayrinhac et al., 2004]. The presence of such latitudinal clines [Endler, 1977], i.e. the gradual decrease of CCRT with latitude, has been identified on different continents [Ayrinhac et al., 2004; Hoffmann & Weeks, 2007]. This independent parallel evolution strongly suggests that the trait is under selection due to the climate. This also holds true for other species in the genus *Drosophila*, where frequent changes between temperate and tropical lifestyle seem to have occurred, and for more distantly related insects [Gibert et al., 2001]. Apart from the geographical origin, CCRT is heavily influenced by plastic changes. In fact, environmental variations affect CCRT to a much

greater extent than genetic differences between populations [Ayrinhac et al., 2004].

1.3 The significance of regulatory evolution

Non-neutral mutations may either be classified as “structural” or “regulatory”. Structural mutations lead to changes in the amino acid sequence of a given protein, thereby possibly altering its conformation and, thus, molecular function. Regulatory mutations, on the other hand, modify spatial, temporal and quantitative patterns of gene expression. The significance of regulatory change in evolution has long been recognized [King & Wilson, 1975; Wray et al., 2003; Whitehead & Crawford 2006; Wray 2007] and is likely brought forth by several advantages of this class of mutations. Since most genes are pleiotropic, i.e. relevant for more than one distinct phenotypic trait, structural mutations that are advantageous in one aspect often have deleterious consequences in another. On the contrary, regulatory mutations theoretically allow for an independent fine tuning of gene expression in different tissues, cell types and developmental stages and in response to various environmental cues. Additionally, the so-called coordinated pleiotropy, i.e. the potential of a transcriptional regulator to influence the expression of a whole set of functionally related genes in a coordinated fashion, and the presumably higher evolvability of promotor structures due to their modularity theoretically further regulatory evolution [Wray et al., 2003]. Consequentially, one can assume that regulatory evolution accounts for a considerable share of the adaptive genetic difference between cold-tolerant and cold-sensitive

populations of *D. melanogaster*. Hence, investigations of the transcriptome, which serves as a link between genotype and phenotype, may provide important insights into the adaptive process and may help to identify particular genes that have undergone regulatory evolution to confer greater cold tolerance.

1.4 Expression analysis via RNAseq

Since the regulation of transcription initiation is the most important factor for the control of gene expression in eukaryotes [Wray et al., 2003], mRNA abundance can be used as a meaningful proxy for overall gene expression. Though, of course, one should keep in mind that the actual amount of functional protein is influenced by factors other than mRNA levels [Lewin, 2000]. The relative abundance of mRNAs can be quantified by qPCR for individual genes and on a genome-wide scale by microarrays [Duggan et al., 1999] and its successor methodology RNAseq [Wang et al., 2009], which provides certain benefits, e.g. more or less absolute transcript quantification, higher accuracy etc.

Briefly, the RNAseq workflow and rationale is as follows: After extraction of total RNA the mRNA-fraction is enriched and reverse-transcribed into cDNA. The resulting cDNA library is then sequenced using next generation sequencing methods resulting in reads with a typical read-length of 30-400 bp. These reads are then mapped to the genome or transcriptome. The number of reads per gene (possibly after correction for gene length) corresponds to the

number of mRNAs of the respective gene, thus providing information about its expression.

Thanks to the well-annotated genome of *D. melanogaster* and established research pipelines, RNAseq data evaluation is relatively straightforward and suffers significantly less drawbacks than comparable projects in non-model organisms.

1.5 Advantages of the study design

Genome-wide expression analyses to uncover regulatory differences between ancestral and derived *D. melanogaster* populations have been mostly conducted at standard lab conditions [Hutter et al. 2008; Müller et al. 2011]. It is, however, anticipated that a huge share of regulatory differences regarding environmental stressors is not apparent under standard conditions, but only becomes detectable when the respective stress, in the present case a cold shock, is applied. Moreover, many transcriptomic studies regarding cold tolerance in *D. melanogaster* have been performed in a single [Qin et al., 2005; Zhang et al., 2011] or few and often old lab strains. Differences in cold tolerance between these strains are likely the result of random genetic drift due to relaxed environmental constraints and inbreeding [Vermeulen et al., 2013] or have been established due to artificial selection [Telonis-Scott et al., 2009]. In contrast, this study employs natural populations of *D. melanogaster* that have spent considerably less time in the laboratory and that differ phenotypically because they have adapted to vastly different environments. To be able to observe a good portion of the adaptive change, populations

were chosen from the climatic extremes of the species range. These populations are vastly different in their CCRT and also represent ancestral and derived cases with respect to the demographic process of range expansion and global dispersal.

A particularly important advantage of the transcriptomic approach in this study is that alternative methods, like, for example, quantitative trait locus (QTL) analyses and genome-wide association studies (GWAS) are limited in a sense, that they require extremely high-resolution and accurate trait quantification before the calling of candidate genes. In the case of CCRT, however, it is highly dubitable if such a resolving power can be achieved. The problem of low signal to noise ratio for the determination of small differences in CCRT is of course still a crucial challenge in the context of this study. But importantly and in contrast to QTL mapping and GWAS, this challenge comes only after the calling of candidate genes during functional validation.

1.6 The importance of functional proof in genetic screens

Genome-wide transcriptomic screens (and alternative methods) can often only provide lists of candidate genes that possibly contain many false positives. Consequentially, the overlap in reported candidate genes for cold tolerance in *D. melanogaster* between different independent studies is considerably small. This is the case when different experimental approaches are used [e.g. Qin et al., 2005; Telonis-Scott et al., 2009; Zhang et al., 2011; Svetec et al., 2011; Wilches et al., 2014; Pool et al., 2016] and even when the same methodology is applied to different fly populations [Mackay et al., 2012; Huang et al., 2012].

To obtain hard evidence that a gene is in fact involved in a certain trait, functional (i.e. phenotypic) proof is highly desirable, but often difficult to come by. This is especially true in the case of an in all likelihood quantitative trait like CCRT that is determined by a multitude of genes with presumably, for the most part, minute individual effect sizes and complex epistatic interactions [Huang et al., 2012]. Further intricacies arise due to the intrinsic and extrinsic variability of CCRT, which render it somewhat imprecise to measure. These properties likely apply to other cold tolerance related traits as well. Consequentially, the acquisition of convincing phenotypic data that demonstrates the involvement of individual genes is often quite laborious and unsuccessful despite considerable effort. Nonetheless, functional evidence is usually the only way a gene can surpass the dubious “candidate” status and, moreover, of importance as proof of concept to give general credence to the employed genetic screening methods.

1.7 Gene knockdown via a genome-wide transgenic RNAi library

As one of the prime model organisms in the field of life sciences *Drosophila melanogaster* has a wide range of genetic tools available for researchers. Of particular use for the functional testing of a whole range of candidate genes is a genome-wide RNAi library [Dietzl et al., 2007], which facilitates the knockdown of almost every gene in the fly genome with relatively little experimental effort. It employs the binary UAS/GAL4-system, consisting of an upstream activating sequence (UAS), i.e. a GAL4-inducible enhancer, and the GAL4 transgene. To achieve the knockdown of an individual gene a strain that

carries a gene specific inverted repeat (IR) under the control of the UAS is crossed to a so-called driver line that expresses GAL4 under the control of a particular promotor. Upon transcription the IR folds into a hairpin structure that induces the RNAi pathway and ultimately leads to the degradation of the corresponding mRNA, thus knocking down target gene expression. Driver lines differ in the promotor that controls GAL4 expression and, furthermore, may contain additional copies of genes that play a part in the RNAi pathway (e.g. *Dicer2*), thereby enhancing RNAi efficiency. The “simplest” driver lines are those with ubiquitously active promotors (e.g. α-Tubulin and Actin5). When these driver lines are used, the knockdown in the respective cross affects all life stages and tissues likewise. Besides, there are conditional promotors that allow for a gene knockdown in specific life stages or tissues, or after a certain stress has been applied.

1.8 Functional data on cold tolerance candidate genes in the literature

Few genes have been functionally implicated in cold tolerance in *D. melanogaster*. Notably, a substantial reduction of the expression of *Hsp70*, which is the gene with the highest upregulation after a cold shock, has been found to have no marked effect on cold tolerance in adult flies [Nielsen et al., 2005] and larvae [Stetina et al., 2015]. A major disruption of cold tolerance after an individual gene knock down has been reported only for *Frost* [Colinet et al., 2010a] and the two small heat shock proteins *Hsp22* and *Hsp23* [Colinet et al., 2010b]. In the example of *Frost*, a substantial increase in CCRT for both female and male flies after a ubiquitous *Frost* knockdown induced by

Actin5 and α-Tubulin driven RNAi activation has been reported [Colinet et al., 2010a]. The knockdown, additionally, resulted in a drastic reduction of cold shock survival 24h after a 12h cold shock [Colinet et al., 2010a]. For *Hsp22* and *Hsp23* all in all similar results were reported [Colinet et al., 2010b]. However, another study completely failed to replicate these results for the gene *Frost* [Udaka et al., 2013]. Thus, functional evidence regarding candidate genes for cold tolerance remains scarce and inconclusive.

2. Results

2.1 CCRT in fly populations from the Netherlands, Denmark, Sweden, Rwanda, Zimbabwe and Zambia

To identify fly strains and populations that evolved particularly diverging cold tolerance phenotypes, CCRT was analyzed after a 7h cold shock (experimental procedure according to [Svetec et al., 2011]) in strains of three tropical African and three temperate European populations in a common garden setting under standard laboratory conditions (21±1°C, see Material & Methods for details). The African populations stem from Lake Kariba, Zimbabwe (population identifier: A), Gikongoro, Rwanda (RG) and Siavonga, Zambia (ZI). The European populations originate from Leiden, the Netherlands (E), Odder, Denmark (DK) and Umea, Sweden (SU). Ambient temperatures are vastly different in the respective locations of origin (see for example www.worldweatheronline.com, which provides detailed climate information for all aforementioned locations). CCRT was always assessed separately for the two sexes. However, differences in CCRT between the sexes are generally marginal after a 7h cold shock (see also Table 1 in Chapter 2.4). Therefore, given CCRT values are usually averaged over female and male flies, unless indicated otherwise. The CCRT of all individual strains of the respective populations is depicted in Figure 1. CCRT displays substantial variation within populations and across replicate experiments within the same inbred strain (Figure 1). Average CCRT values of the Rwandan, Zambian, Danish, and Swedish population are depicted in Figure 2.

A157 and E14 are the only strains tested from the Zimbabwean and Dutch population (Figure 1). They have been included because their particularly fast and particularly slow chill coma recovery, respectively, has been demonstrated previously [Svetec et al., 2011]. Additionally, they have been formerly employed for QTL analyses to identify candidate genes affecting cold tolerance [Svetec et al., 2011; Wilches et al., 2014].

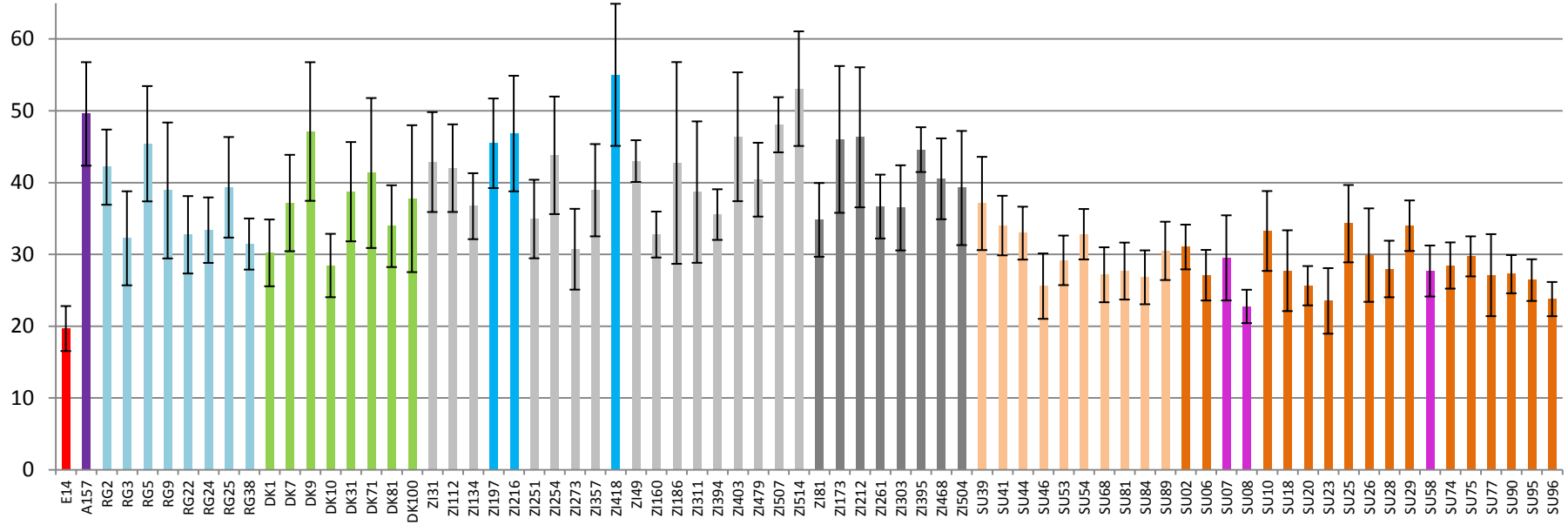

Figure 1 | CCRT of individual strains
The y-axis shows chill coma recovery time (CCRT) in minutes. On the x-axis are the different strains: red: E14 (Leiden, the Netherlands), purple: A157 (Kariba, Zimbabwe), light blue: RG (Gikongoro, Rwanda), green: DK (Odder, Denmark), grey: ZI (Siavonga, Zambia), orange: SU (Umea, Sweden); different shading within the Zambian and Swedish population denotes different inbreeding status, strains from these populations chosen for further analysis are highlighted in turquoise (ZI197, ZI216, ZI418) and pink (SU07, SU08, SU58); choice was also based on criteria other than CCRT such as sequence availability. CCRT is calculated as the time flies need to recover after being brought back to room temperature following a 7h cold shock in an ice water bath. Depicted values are averaged over several (5-20) independent replicate experiments and over both sexes. Error bars denote the standard deviation.

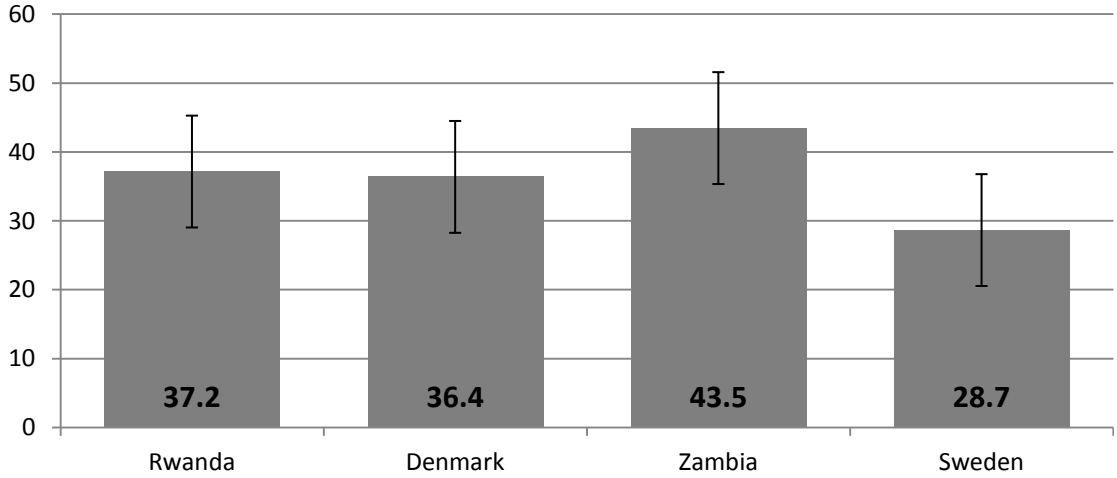

Figure 2 | CCRT of populations
The y-axis shows chill coma recovery time (CCRT) in minutes. CCRT is calculated as the time flies need to recover after being brought back to room temperature following a 7h cold shock in an ice water bath. Depicted values are averaged over 8 (Rwanda & Denmark) or ~30 (Zambia & Sweden) strains per population, several independent experimental replicates and both sexes. Error bars denote the standard deviation.

Results demonstrate the importance of population phenotyping, as one cannot always infer differences in cold tolerance from climatic distinctions. For example, there is unexpectedly little difference between the Rwandan flies from Gikongoro (RG) and the Danish flies from Odder (DK). This may be, amongst other things, explained by the high altitude (1927 m above sea level) of Gikongoro. Additionally, factors other than climate may influence cold tolerance. The Rwandan population also displays a relatively high level of European admixture. The lines assessed here, however, were explicitly chosen on the basis of the criterion that they are almost free from genomic regions of European ancestry.

On the other hand, the populations from Zambia and Sweden display substantial differences in CCRT with little overlap in the recovery times of individual strains (Figure 1).

2.2 CCRT under varying environmental conditions and the importance of phenotypic plasticity

Phenotypic plasticity (see [Forsman, 2015] for a recent review) is commonly defined as the ability of a single genotype to generate multiple different phenotypes in dependence of environmental variations. This ability is considered especially important for insects with short generation times, like e.g. *Drosophila*, in temperate regions with vastly differing environmental regimes throughout the course of the year. Consequently, it has been previously shown that the environment strongly influences cold tolerance, as determined via CCRT, in *D. melanogaster* and even that the relative importance of plastic changes greatly exceeds genetic population differentiation [Ayrinhac et al., 2004]. In an effort somewhat similar to this study the effects of cold rearing (17°C vs. 22°C), an acclimation treatment (12h at 6°C prior to the CS vs. no acclimation), and a combination of the two on CCRT were tested in ten Swedish and ten Zambian strains. Additionally, the relative contributions of genetic and environmental factors on the observed variation in CCRT were likewise assessed via a multifactorial ANOVA (Table 1). In both populations cold rearing and the acclimation treatment substantially decrease CCRT (Figure 3). The plastic changes elicited by cold rearing and the acclimation treatment appear to be at least partially independent, since CCRT is most strongly reduced when flies are both reared at 17°C and subjected to the acclimation treatment. The effects of cold rearing, however, are more pronounced (Figure 3, Table 1). Importantly, differences between the populations persist over all four environmental

regimes. Thus, the effects of hereditary adaptation, cold rearing temperature and the acclimation treatment are additive, while sex has no significant influence on CCRT under the tested conditions (Table 1).

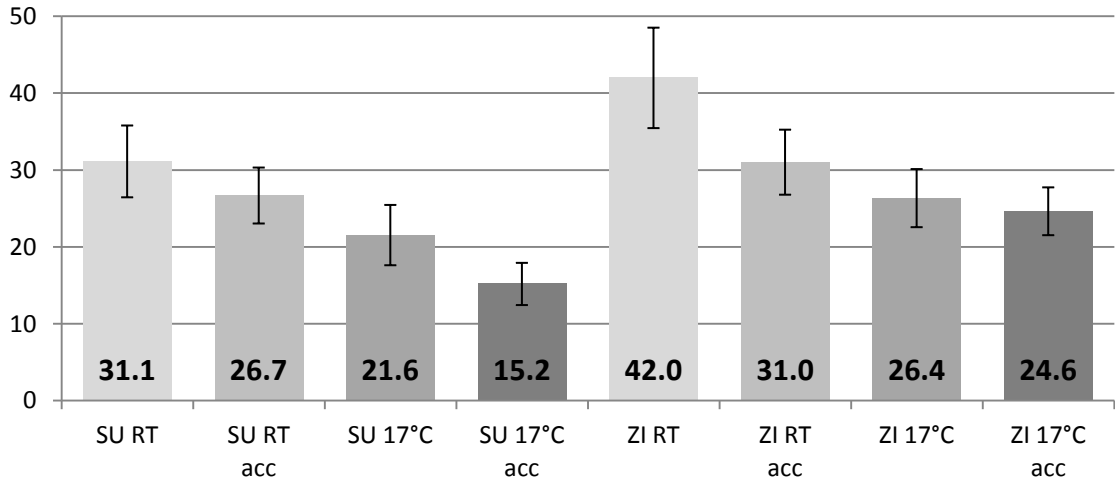

Figure 3 | The effect of cold rearing and acclimation on CCRT
The y-axis shows chill coma recovery time (CCRT) in minutes after a 7h cold shock, on the x-axis are the different treatment combinations for the Swedish (SU) and Zambian (ZI) population. RT / 17°C = 22°C / 17°C rearing temperature, acc = acclimation treatment (6°C for 12h before the cold shock). Depicted values are averaged over 10 strains per population, 4 independent experimental replicates per treatment and ~10 flies of each sex per repeat. Thus, every column represents the average CCRT of ~800 flies. Error bars denote the standard deviation.

Table 1 | Relative contribution and significance of factors influencing CCRT according to a multifactorial ANOVA

Factor	P-value	variation explained
Population (Sweden vs. Zambia)	1.5E-14	22.2%
Sex	2.7E-01	0.3%
Rearing temperature (17°C vs. 22°C)	2.2E-16	48.6%
Acclimation (12h 6°C vs. no acclimation)	4.1E-12	16.2%
Error	n.a.	12.7%

2.3 CCRT after repeated cold shocks

In another set of experiments, the changes in CCRT after repeated cold shocks were tested in the Rwandan and Danish population and in the Zimbabwean strain A157 and the Dutch strain E14. To this end, flies were returned to food vials after the first CCRT experiment and shocked again on the following day. For the Danish flies and for A157 and E14 this procedure was repeated twice. Accordingly, flies spent 17h hours at room temperature in between the subsequent 7h cold shocks. In the Rwandan population flies recover slightly faster after the second cold shock, whereas in the Danish population recovery time gradually increases after each subsequent cold shock (Figure 4). The total differences in CCRT, however, are relatively small.

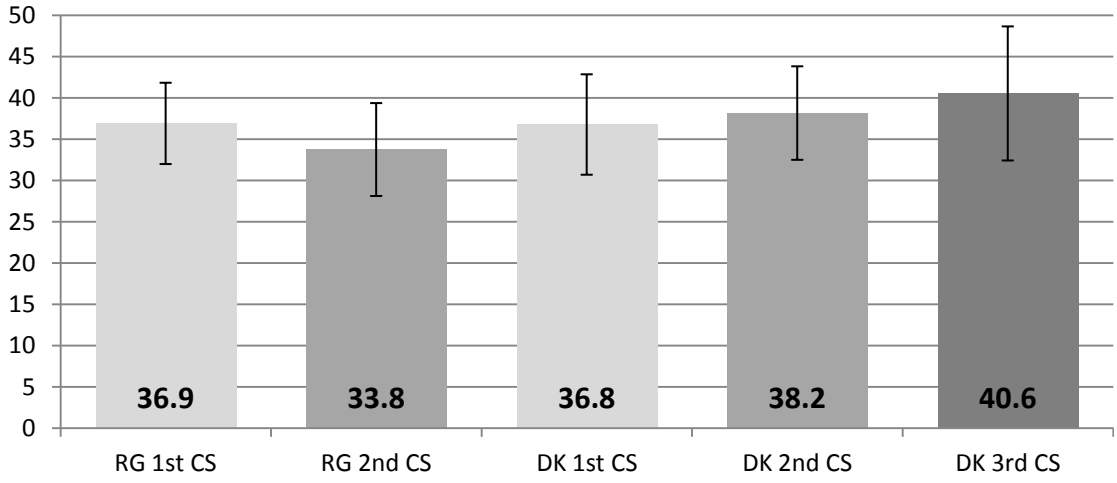

Figure 4 | CCRT after repeated cold shocks for Rwanda and Denmark
The y-axis shows chill coma recovery (CCRT) in minutes. Flies stem from Gikongoro, Rwanda (RG) and Odder, Denmark (DK). They were subjected to repeated 7h cold shocks (CS) with intermittent recovery periods of 17h in food vials at room temperature. Values are averaged over eight independent experimental replicates with mostly eight strains per population and ~10 flies of each sex per repeat. Thus, every column represents the average CCRT of ~1200 flies. Error bars denote the standard deviation.

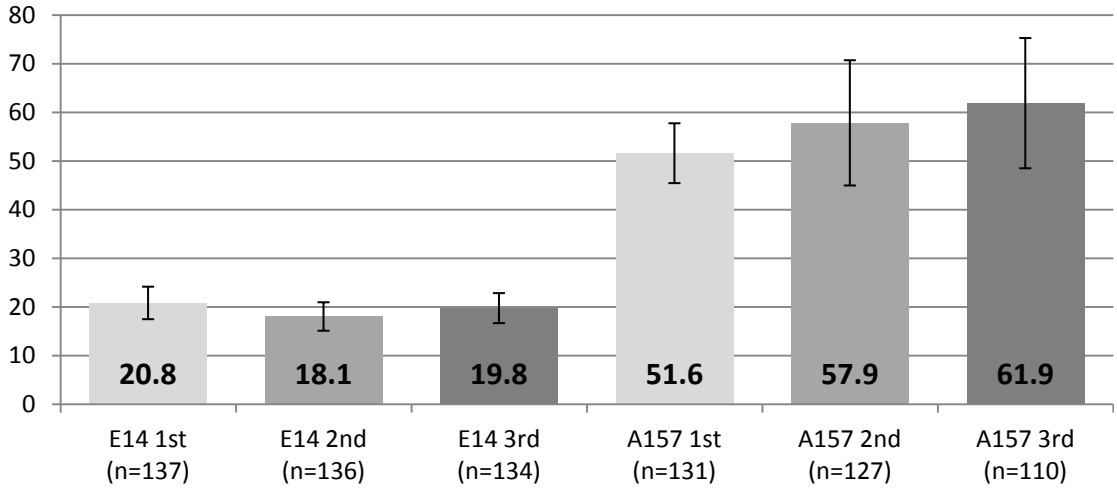

Figure 5 | CCRT after repeated cold shocks for E14 and A157
The y-axis shows chill coma recovery (CCRT) in minutes. Flies stem from Leiden, the Netherlands (E14) and Lake Kariba, Zimbabwe (A157). They were subjected to repeated 7h cold shocks (CS) with intermittent recovery periods of 17h in food vials at room temperature. Values are averaged over seven independent experimental replicates (n = total number of flies) and both sexes. Error bars denote the standard deviation.

E14 shows a slightly diminished CCRT after the second and third cold shock (Figure 5). Again, the differences in recovery time are relatively small. In A157, however, CCRT increases by roughly five minutes after every subsequent cold shock (Figure 5). Likewise, there is an increase in mortality, especially in female flies where it reaches 20% between the second and third cold shock. This gradual decline in cold shock recovery and survival might be another characteristic of the limited cold tolerance of tropical strains beside their higher initial CCRT. However, A157 is the only truly tropical strain for which the response to repeated cold shocks was evaluated.

In all other tested strains apart from A157, the incidence of death increases only marginally during subsequent cold shocks, but this could also be partially due to the stress associated with the transfer of flies (i.e. repeatedly knocking them from one vial to another, which sometimes results in flies getting stuck in

the food etc). Overall, the great majority of flies survive multiple cold shocks and CCRT values do not change drastically. These results corroborate the fully reversible nature of chill coma and demonstrate the potential relevance of chill coma recovery in nature, which, in principle, enables flies two withstand several cold nights in a row.

2.4 Life history traits and potential cold tolerance related trade offs

Two distinct traits of an organism are frequently not independent of one another. This means that a (beneficial) increase in one trait may result in a (detrimental) decrease in the other due to certain constraints. This phenomenon is called a trade off (see [Garland, 2014] for a short explanatory introduction).

To assess general fitness parameters of European and African flies the egg laying capacity in a 24h time interval (Figure 6) and the subsequent egg-to-larva- and larva-to-imago-viability (Figures 7&8) were evaluated. Additionally, CCRT experiments with eclosed adult flies from these experiments (Figure 9) were performed. Finally, long term survival of experimental male flies, with or without having experienced a single 7h cold-shock, was monitored (Figure 10). European flies perform consistently and substantially better in all these tests (Figures 10 & 11).

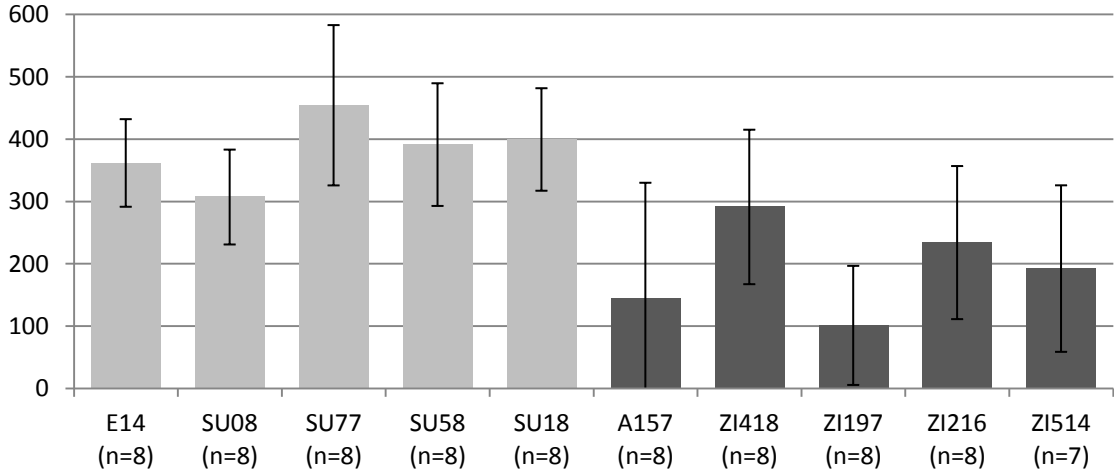

Figure 6 | Oviposition rates of European and African *D. melanogaster*
The y-axis shows the mean number of eggs laid by 15 young females during a 24h time span. Female flies were kept in a mating cage together with 15 young males on a molasses plate. Oviposition was recorded after three days of acclimatization. Flies stem from temperate Europe (E, SU) and tropical Africa (A, ZI). Values are averaged over a number of independent experimental replicates (n). Error bars denote the standard deviation.

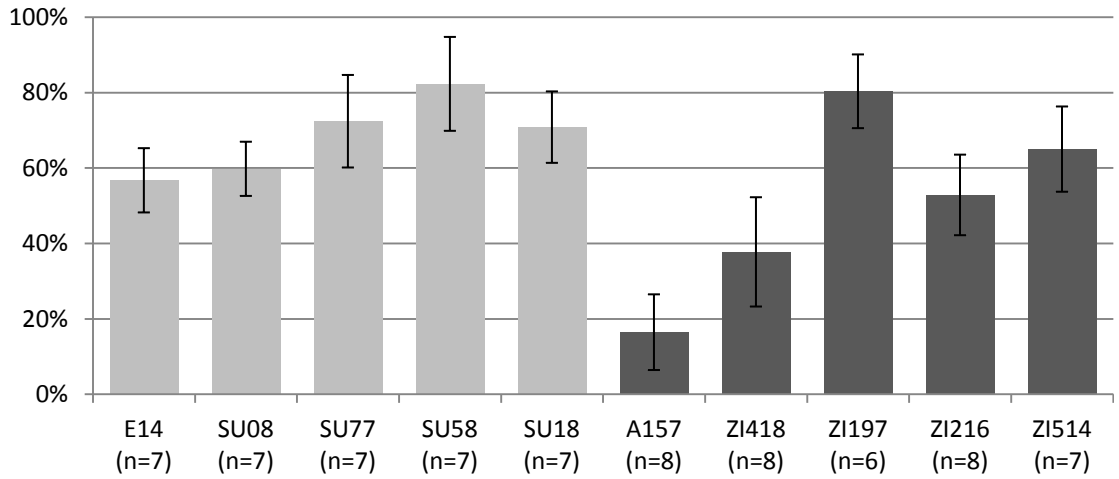

Figure 7 | Hatching rates of European and African *D. melanogaster*
The y-axis shows the percentage of hatched larvae from the total number of eggs five days after oviposition. Flies stem from temperate Europe (E, SU) and tropical Africa (A, ZI). Values are averaged over a number of independent experimental replicates (n). Error bars denote the standard deviation.

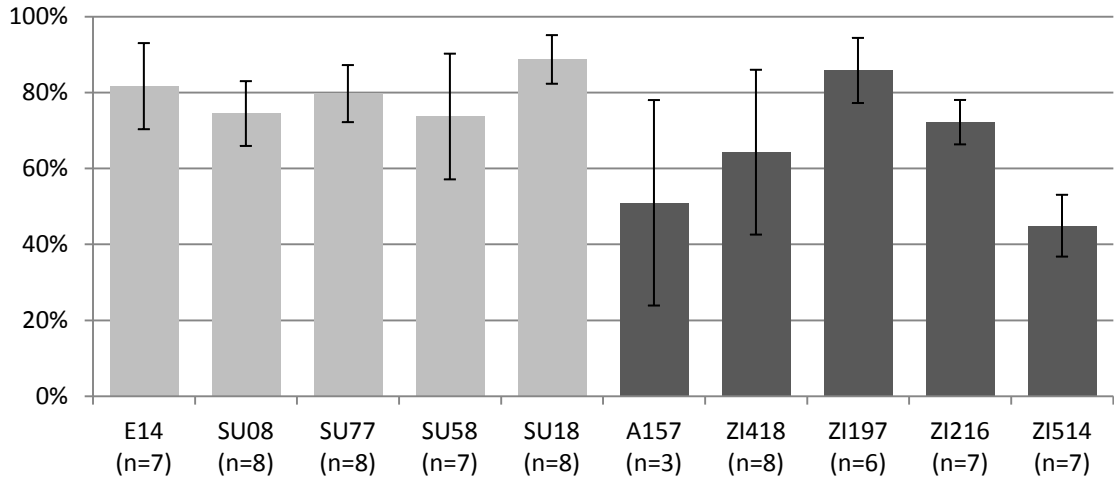

Figure 8 | Eclosion rates of European and African *D. melanogaster*
The y-axis shows the percentage of eclosed flies 22 days after oviposition out of ~50 larvae picked and transferred to a fresh vial on day 5. Flies stem from temperate Europe (E, SU) and tropical Africa (A, ZI). Values are averaged over a number of independent experimental replicates (n). Error bars denote the standard deviation.

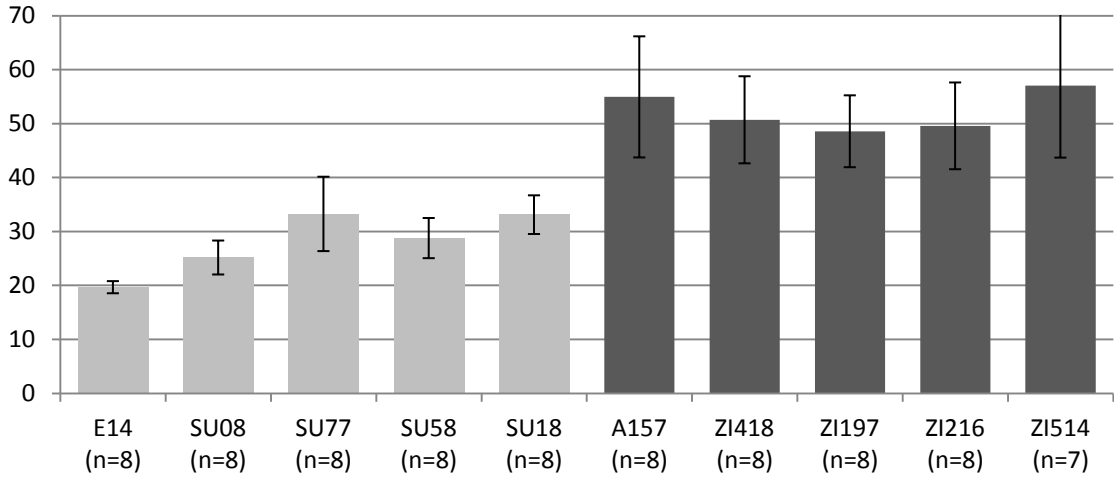

Figure 9 | CCRT of European and African *D. melanogaster*
The y-axis shows chill coma recovery time (CCRT) in minutes. CCRT is calculated as the time flies need to recover after being brought back to room temperature following a 7h cold shock in an ice water bath. CCRT was assessed 19 days after oviposition. Flies stem from temperate Europe (E, SU) and tropical Africa (A, ZI). Values are averaged over a number of independent experimental replicates (n). Error bars denote the standard deviation.

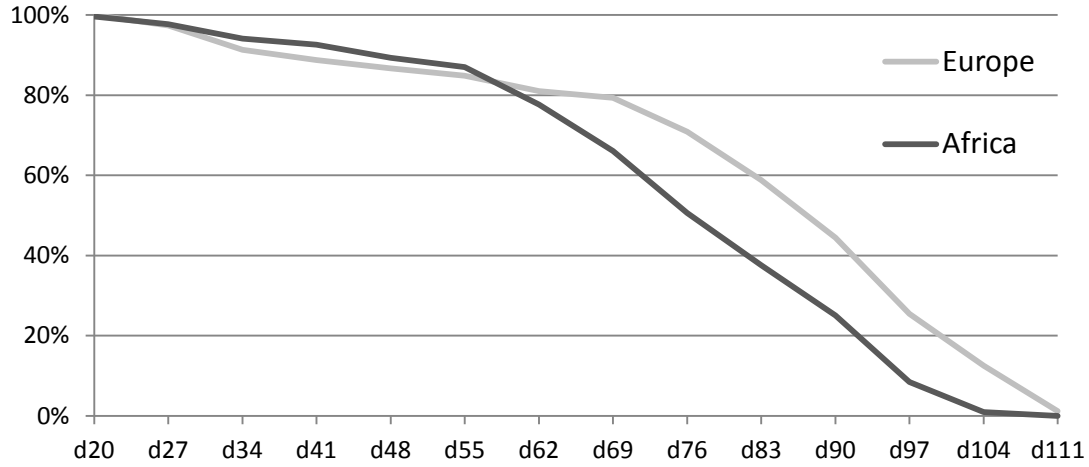

Figure 10 | Longevity of European and African *D. melanogaster*
The y-axis shows the percentage of living flies. The x-axis shows the days after oviposition. Flies stem from temperate Europe (E, SU) and tropical Africa (A, ZI). Values are calculated with 711 (Europe) and 524 (Africa) males out of five strains per population and out of ~14 independent repeats per strain with ~10 male flies per repeat. ~50% of the flies were subjected to a single cold shock before. No difference between shocked and unshocked flies was observed.

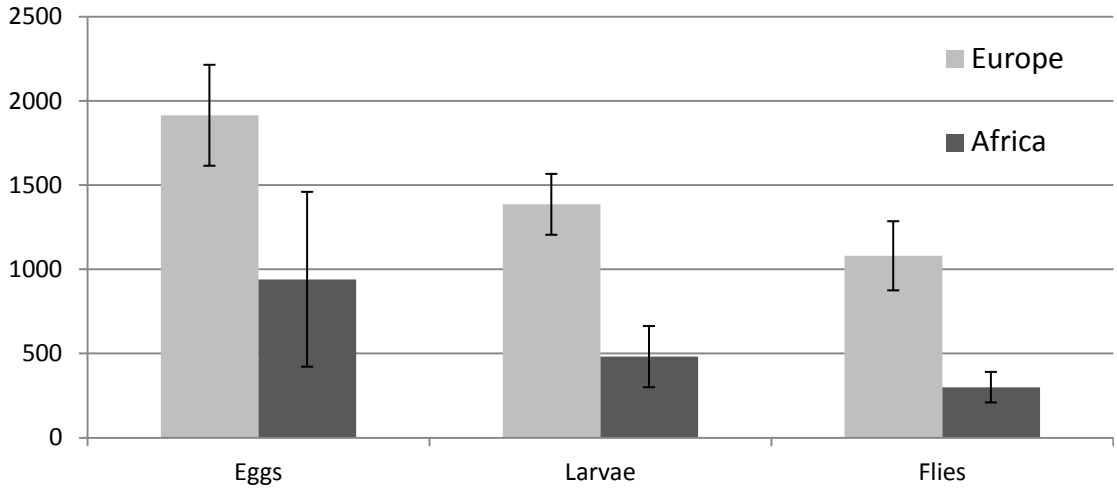

Figure 11 | Reproductive output of 5 European and 5 African strains
The y-axis shows the number of eggs and the number of larvae and flies that developed from these. Eggs = number of eggs laid by 15 young females during a 24h time span. Larvae = number of larvae to develop from these eggs within 5 days after oviposition. Flies = calculated value (mean percentage of ~50 picked larvae that developed into adult flies 22 days after oviposition times the total number of hatched larvae). All three values are given as the sum of five strains per population. Values are averaged over eight (eggs) or seven (larvae & flies) independent experimental replicates. Error bars denote the standard deviation.

Due to the high variation in all measured life history traits it is not possible to establish a meaningful correlation of general fitness traits and CCRT within the respective populations. However, both CCRT and life history traits clearly demonstrate increased vigor of European in contrast to African flies. These results are likely the consequence of suboptimal conditions for tropical strains in a standard lab environment. Especially humidity seems to be an important factor. One of the eight replicate experiments was characterized by particularly high humidity, which even resulted in dew droplets on the agar plates used for oviposition. In this particular replicate the African flies laid substantially more eggs than in the other replicate experiments, albeit still less than the European flies (data not shown). It is noteworthy in this regard, that although experimental conditions could not be kept completely constant due to limited fly housing facilities, each replicate experiment was conducted in parallel and, thus, at identical conditions for European and African flies. In conclusion, the results provide no evidence for a trade off between cold tolerance and reproductive success or longevity.

2.5 qPCR for eight candidate genes following a cold shock in one European and one African strain

Eight genes were chosen that have been previously shown to change their expression in response to cold temperatures or have been otherwise implicated in cold tolerance:

Frost [Goto, 2001; Colinet et al. 2010a, Qin et al., 2005],

Hsp23 [Qin et al., 2005; Colinet et al., 2010c],

brinker [Wilches et al., 2014],

smp30 [Goto, 2000],

TotA [Zhang et al., 2011],

TotC [Zhang et al., 2011],

CG10912 [Qin et al., 2005; Zhang et al., 2011],

CG12164 [Graveley et al., 2011].

For these genes expression was analyzed via quantitative real time PCR (qPCR) in one Swedish (SU08) and one Zambian (ZI418) strain on a fine timescale: before the cold shock, 3.5h into the cold shock, right at the end of the 7h cold shock, 15, 30, 45, 60, 90, 120, and 240 minutes after the cold shock. Additional samples were taken at the timepoint of 50% recovery (20+6 and 43+12 minutes recovery + handling time for SU08 and ZI418, respectively), separately for recovered and unrecovered flies. Results are depicted in Figures 12-17.

For *Frost*, *Hsp23* and *CG10912* results show a strong upregulation upon cold shock with a peak around 90 - 120 minutes in the recovery phase (Figures 12-14). For the other genes results are less clear. *brinker* reaches a peak of

upregulation with almost twofold expression 30 minutes after the cold shock (Figure 15). *smp-30* appears to be slightly downregulated during early recovery and slightly upregulated late in recovery (Figure 16). Expression of *CG12164* is relatively constant at all conditions but always higher in African flies (Figure 17). Results for *TotA* & *TotC* (not shown) are inconclusive, because of huge variations between biological replicates, but show a high correlation of expression in identical samples. Notably, expression of all eight genes is virtually independent of recovery status as recovered and unrecovered flies display very similar transcript amounts at the timepoint when 50% of flies have recovered. Expression during the cold shock remains, in general, unchanged compared to RT and differences between the populations are quantitative rather than qualitative for the examined genes.

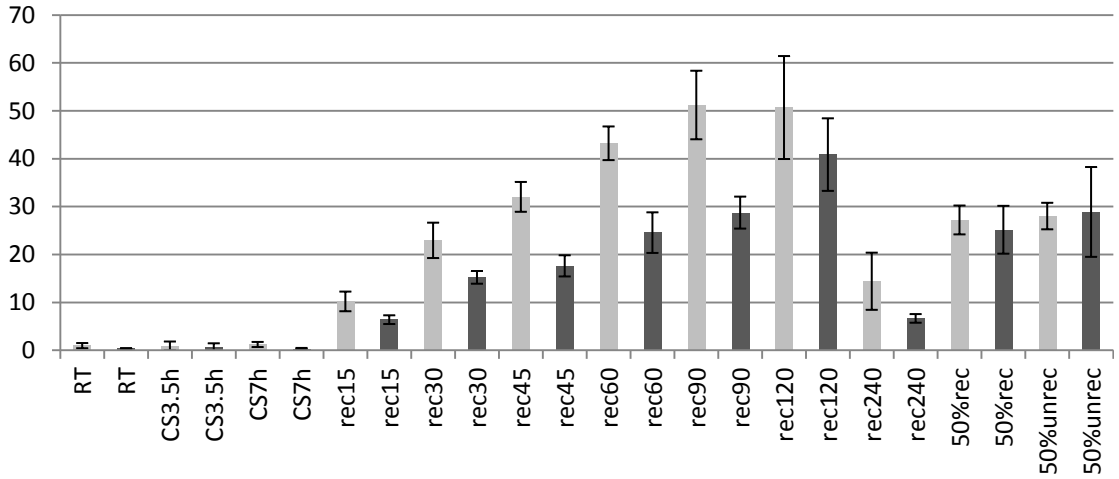

Figure 12 | Expression of *Frost*
The y-axis shows expression relative to the first column (SU08 at RT), which is set to one. Expression values are calculated as the geometric mean of four biological replicates. SU08(Sweden) = light gray, ZI418(Zambia) = dark gray; RT = room temperature, CS = during cold shock, rec = during recovery (time in minutes), 50%rec/unrec = at the timepoint when 50% of flies had recovered (with handling time to transfer flies, this equals 26 minutes for SU08 and 55 minutes for ZI418) separately for recovered and unrecovered flies. Error bars denote the standard deviation.

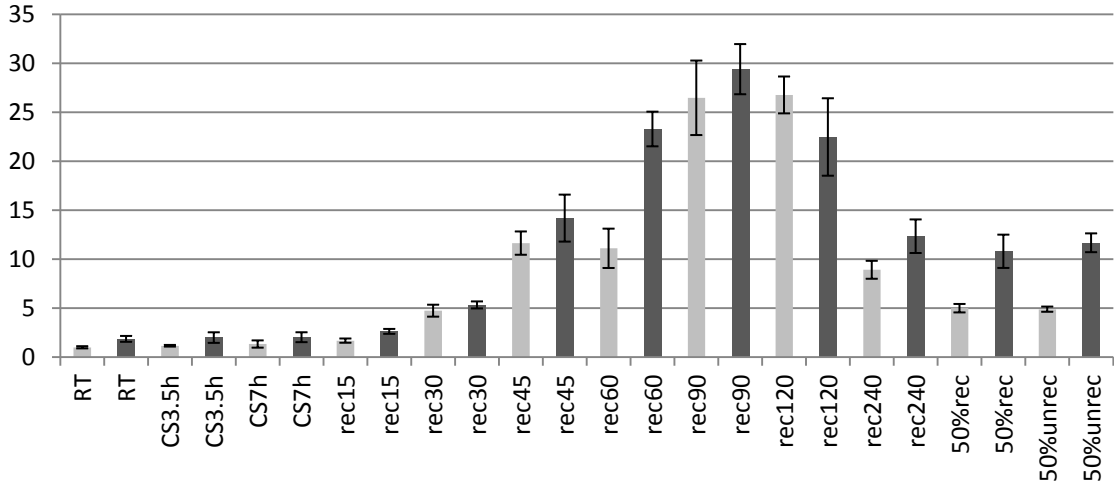

Figure 13 | Expression of *Hsp23*
The y-axis shows expression relative to the first column (SU08 at RT), which is set to one. Expression values are calculated as the geometric mean of four biological replicates. SU08(Sweden) = light gray, ZI418(Zambia) = dark gray; RT = room temperature, CS = during cold shock, rec = during recovery (time in minutes), 50%rec/unrec = at the timepoint when 50% of flies had recovered (with handling time to transfer flies, this equals 26 minutes for SU08 and 55 minutes for ZI418) separately for recovered and unrecovered flies. Error bars denote the standard deviation.

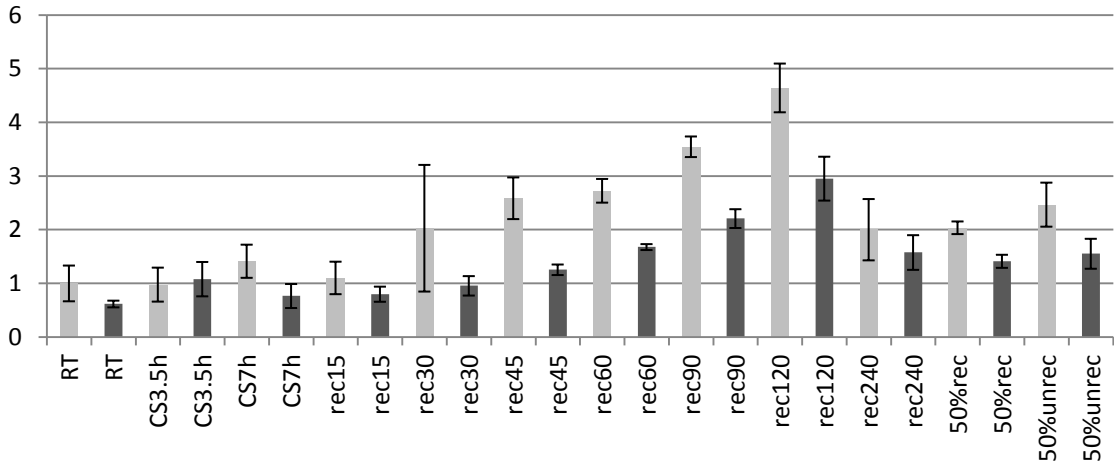

Figure 14 | Expression of *CG10912*
The y-axis shows expression relative to the first column (SU08 at RT), which is set to one. Expression values are calculated as the geometric mean of four biological replicates. SU08(Sweden) = light gray, ZI418(Zambia) = dark gray; RT = room temperature, CS = during cold shock, rec = during recovery (time in minutes), 50%rec/unrec = at the timepoint when 50% of flies had recovered (with handling time to transfer flies, this equals 26 minutes for SU08 and 55 minutes for ZI418) separately for recovered and unrecovered flies. Error bars denote the standard deviation.

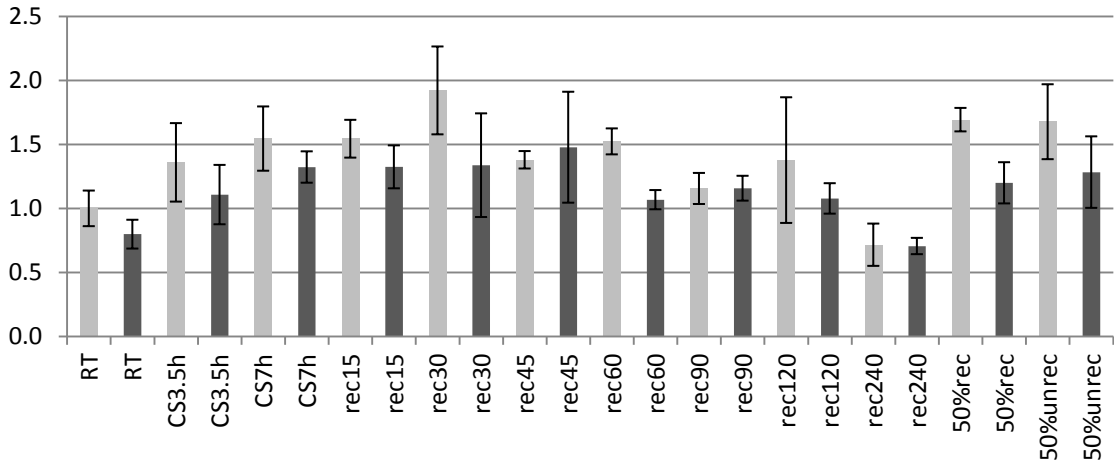

Figure 15 | Expression of *brinker*
The y-axis shows expression relative to the first column (SU08 at RT), which is set to one. Expression values are calculated as the geometric mean of four biological replicates. SU08(Sweden) = light gray, ZI418(Zambia) = dark gray; RT = room temperature, CS = during cold shock, rec = during recovery (time in minutes), 50%rec/unrec = at the timepoint when 50% of flies had recovered (with handling time to transfer flies, this equals 26 minutes for SU08 and 55 minutes for ZI418) separately for recovered and unrecovered flies. Error bars denote the standard deviation.

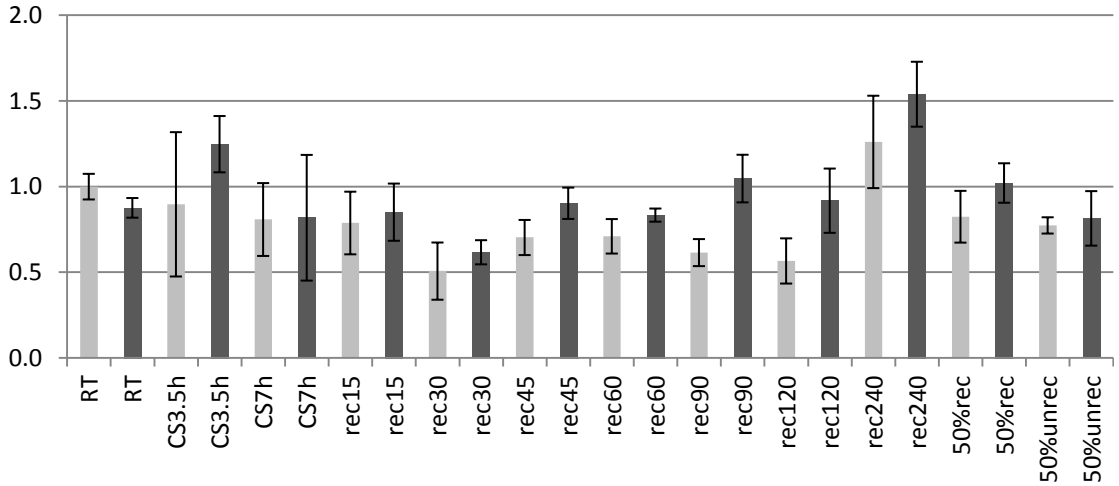

Figure 16 | Expression of *smp30*
The y-axis shows expression relative to the first column (SU08 at RT), which is set to one. Expression values are calculated as the geometric mean of four biological replicates. SU08(Sweden) = light gray, ZI418(Zambia) = dark gray; RT = room temperature, CS = during cold shock, rec = during recovery (time in minutes), 50%rec/unrec = at the timepoint when 50% of flies had recovered (with handling time to transfer flies, this equals 26 minutes for SU08 and 55 minutes for ZI418) separately for recovered and unrecovered flies. Error bars denote the standard deviation.

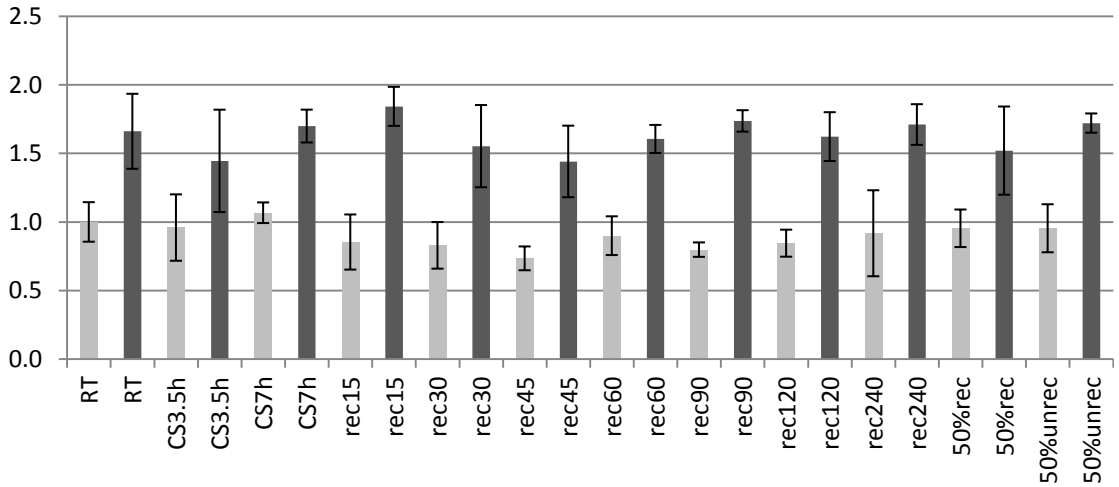

Figure 17 | Expression of *CG12164*
The y-axis shows expression relative to the first column (SU08 at RT), which is set to one. Expression values are calculated as the geometric mean of four biological replicates. SU08(Sweden) = light gray, ZI418(Zambia) = dark gray; RT = room temperature, CS = during cold shock, rec = during recovery (time in minutes), 50%rec/unrec = at the timepoint when 50% of flies had recovered (with handling time to transfer flies, this equals 26 minutes for SU08 and 55 minutes for ZI418) separately for recovered and unrecovered flies. Error bars denote the standard deviation.

2.6 RNAseq in four temperate and four tropical strains following a cold shock

High-throughput RNA sequencing (RNAseq) of mRNA extracted from whole male flies was performed at four different experimental conditions: before, during and 15 & 90 minutes in the recovery phase after a 7h cold-shock. Whereas the timepoint before the cold shock serves as a baseline control, the 15 minutes timepoint was chosen, because some of the expression change at this time might be directly related to the process of recovery itself, since this is also the timepoint when the first fast-recovering European flies tend to wake up from their cold-induced coma [Svetec et al., 2011]. The 90 minute timepoint, on the other hand, was chosen, because the preceding qPCR experiments showed that the genes that are known to strongly respond towards a cold shock peak in expression around this timepoint (Chapter 2.5, but see also [Colinet et al., 2010a; Colinet et al., 2010b; Colinet et al., 2010c]). RNAseq was performed on three Swedish strains with particular short CCRT and on three Zambian strains with particular long CCRT. To broaden the scope, one additional fast-recovering European strain from the Netherlands and one additional slow-recovering African strain from Zimbabwe were included. The latter two have been previously employed in QTL analyses to identify genes affecting cold tolerance [Svetec et al., 2011; Wilches et al., 2014]. The CCRT of all eight strains is shown in Figure 18.

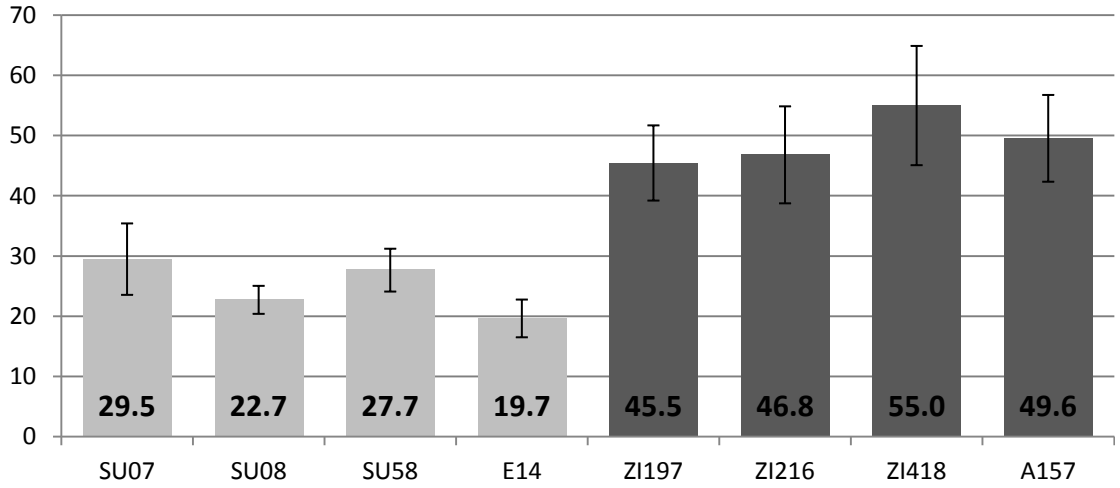

Figure 18 | CCRT for the eight focal strains
The y-axis shows chill coma recovery time (CCRT) in minutes. CCRT was determined following a 7h cold shock in an ice-water bath. Depicted values are averaged over both sexes and a multitude of independent experiments. Strains originate from Umea, Sweden (SU07, SU08, SU58), Leiden, the Netherlands (E14), Siavonga, Zambia (ZI197, ZI216, ZI418), and Lake Kariba, Zimbabwe (A157). Error bars denote the standard deviation. Modified from [von Heckel et al., 2016].

Exploring the transcriptomic data, genes that are differentially expressed due to either experimental condition or due to continental origin, and, most interestingly, genes that respond to the cold shock in a population-specific way, i.e. genes that exhibit a genotype by environment interaction (GEI) were determined. To my knowledge, this is the first study in which the genome-wide transcriptional response to a cold shock is measured and compared between a derived cold tolerant European and an ancestral cold sensitive African population of *D. melanogaster*.

Transcriptome overview

To investigate population differences in the transcriptional cold shock response, five day old male flies of the four African and the four European

strains were subjected to a 7h cold shock. Total RNA was isolated from whole flies of each strain at four distinct timepoints: before the cold shock (RT), 3.5h into the cold shock (CS) and 15 & 90 minutes after flies have been brought back to room temperature (rec15, rec90). After library preparation and sequencing, in total, over 1.8 billion 51 bp reads were obtained from 64 cDNA libraries, which comprise two biological replicates of each strain-and-timepoint-combination. Read quality is generally very high, with a mean Phred score of 35.7. Notably, for the first and last positions the mean Phred score does not drop below 30. Overall, more than 90% of the reads map to annotated transcripts, just under 4% map to rRNA, about 1% to other noncoding RNAs, and a little over 4% of all reads could not be mapped to the *D. melanogaster* genome. Of the 13,955 annotated protein-coding genes in FlyBase release 5.57 [St Pierre et al., 2014], 13,821 have at least one mapped read in at least one library, whereas 12,617 genes have at least one mapped read in every library.

A principle component analysis (PCA) [Pearson, 1901] demonstrates tight clustering of biological replicates and related samples and reveals ample differences between populations and conditions (Figure 19). The first principle component accounts for 23% of the total variance and clearly separates the different conditions with the exception of RT and CS. The second principal component separates the African from the European samples and accounts for 18% of the total variance. Since the Dutch and the Zimbabwean strain strongly resemble the Swedish and Zambian strains, respectively, all

European and all African strains were treated as a single population in most subsequent analyses. However, all of these analyses were also performed with only the Swedish and Zambian lines (results not shown) without strong effects on the outcome.

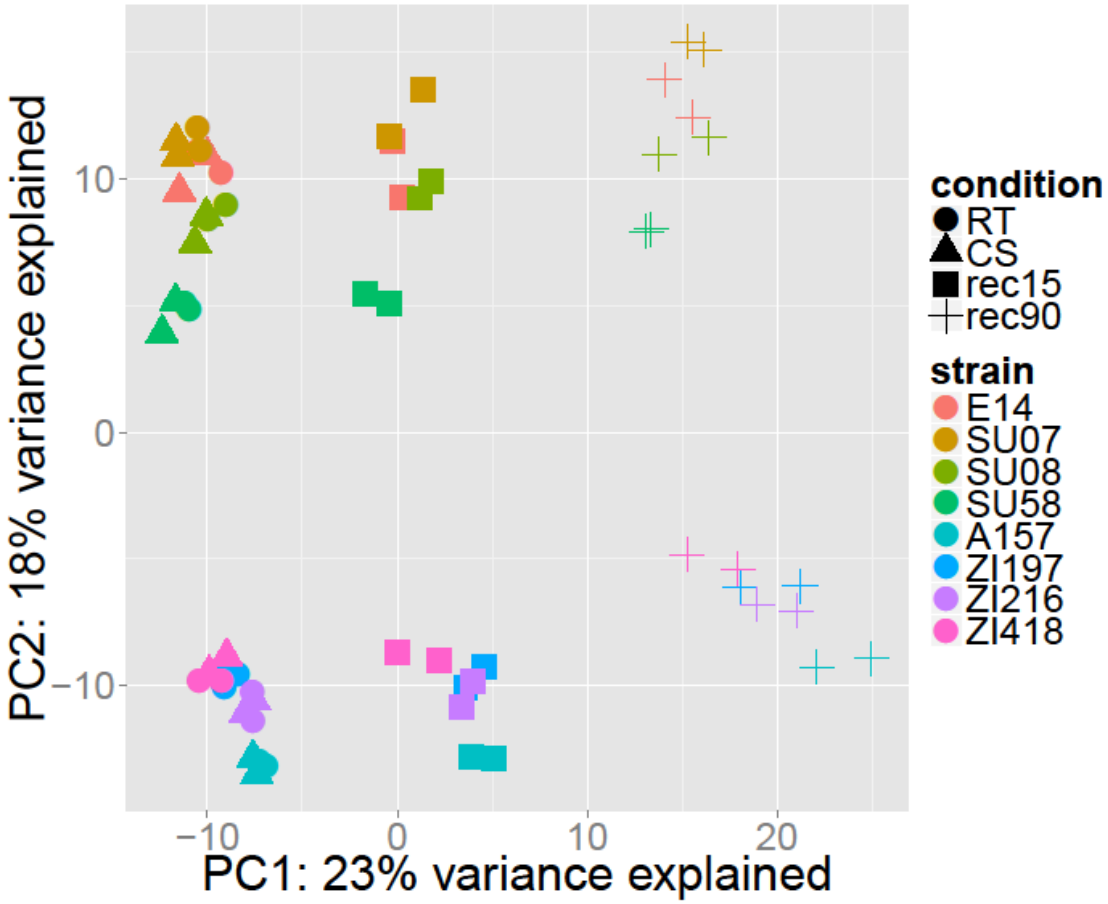

Figure 19 | Transcriptome overview: PCA
PCA (principal component analysis) was calculated using the built-in methods provided by DESeq2 [Love et al., 2014] for variance stabilizing transformation of read counts and PCA on the 500 genes with the highest overall expression variance. RT = room temperature, CS = 3.5h cold shock, rec15/90 = 15/90 minutes after a 7h cold shock. Strains originate from Umea, Sweden (SU07, SU08, SU58), Leiden, the Netherlands (E14), Siavonga, Zambia (ZI197, ZI216, ZI418), and Lake Kariba, Zimbabwe (A157). Note that samples are clearly separated according to continent and condition with the exception of RT and CS samples, which cluster tightly together in both populations such that symbols partly overlap. Published in [von Heckel et al., 2016].

Moreover, the RNAseq results are in very good agreement to the aforementioned qPCR results (Chapter 2.5) for eight genes in one Swedish and one Zambian strain, thus corroborating the accuracy of the produced gene expression data. A direct comparison between the RNAseq and qPCR results is shown in Table 2.

Table 2 | Comparison of gene expression in RNAseq and qPCR

Gene	Strain	Contrast	L2FC qPCR	L2FC RNAseq
Frost	SU08	rec15 vs. RT	3.40	2.45
		rec90 vs. RT	5.63	4.61
	ZI418	rec15 vs. RT	4.09	4.44
		rec90 vs. RT	6.14	7.03
Hsp23	SU08	rec15 vs. RT	0.75	0.63
		rec90 vs. RT	4.73	4.09
	ZI418	rec15 vs. RT	0.50	0.26
		rec90 vs. RT	3.98	4.01
CG10912	SU08	rec15 vs. RT	0.14	0.03
		rec90 vs. RT	1.83	1.33
	ZI418	rec15 vs. RT	0.37	0.13
		rec90 vs. RT	1.84	1.59
brinker	SU08	rec15 vs. RT	0.63	0.92
		rec90 vs. RT	0.21	0.37
	ZI418	rec15 vs. RT	0.73	0.47
		rec90 vs. RT	0.54	-0.30
smp-30	SU08	rec15 vs. RT	-0.34	-0.09
		rec90 vs. RT	-0.70	-0.50
	ZI418	rec15 vs. RT	-0.04	-0.45
		rec90 vs. RT	0.26	-0.67
CG12164	SU08	rec15 vs. RT	-0.24	0.41
		rec90 vs. RT	-0.37	-0.21
	ZI418	rec15 vs. RT	0.14	-0.05
		rec90 vs. RT	0.02	-0.26

For *TotA* & *TotC*, which were also measured via qPCR, expression is extremely inconsistent between biological replicates and appears to be irrespective of condition in both the qPCR and the RNAseq data.

Global expression differences between conditions

In order to identify the common properties of the cold shock response, the numbers of mapped reads for each gene in the three cold treatments (CS, rec15, rec90) were compared to the respective numbers at RT across all eight strains. For CS, in general, only minute changes in gene expression are apparent, which probably reflects the strong reduction in overall transcription at ~0°C. Still, 38 genes show consistent, if only moderate downregulation at a 5% FDR-cutoff ([von Heckel et al., 2016] Table S3). These genes are functionally enriched for being involved in the (negative) regulation of cellular metabolism and for being located in the nucleus. Prominent examples hereof are the genes *hairy* and *extramacrochaetae*, which are named according to their role in bristle patterning, but play a part in a wide variety of physiological and developmental processes via protein dimerizing with a range of transcription factors and thus abolishing their DNA binding capability [Costa et al., 2014]. In contrast, not a single gene is found to be upregulated at the CS timepoint.

The most striking characteristic of the cold shock response in the recovery phase is the swift and massive increase in the expression of molecular chaperones. Already 15 minutes after the end of the cold shock several heat shock proteins (Hsp) are strongly upregulated compared to their expression at RT. This is in line with similar findings in previously published studies (e.g. [Colinet et al., 2010c]) and equally true for African and European flies. The gene with the highest fold-change is *Hsp70* (the six copies of *Hsp70* are

treated as a single gene, see also Material & Methods, Ambiguous mapping) with a roughly 60-fold increase in expression at rec15. This is accompanied by a more than 4-fold upregulation of *DNAJ1*/*Hsp40* and *starvin*, which are both known to closely interact with *Hsp70* at the protein level [Fan et al., 2003; Takayama et al., 1999]. In total, 364 and 518 genes are significantly up- and downregulated, respectively, at rec15 ([von Heckel et al., 2016] Tables S4 & S5). For these gene sets, the majority of GO enrichment found at a significance cutoff of 5% is for the set of upregulated genes. Here, a few stress/stimulus response terms, which mostly are driven by Hsps, and a few broader terms related to regulation and development are enriched in the category “biological process”. For the downregulated genes the only significantly enriched GO term is “RNA export from nucleus”.

At rec90, 1535 genes are higher expressed than at RT and 1979 genes are less expressed ([von Heckel et al., 2016] Tables S6 & S7). Again, many genes that are highly upregulated belong to the Hsp gene family and the list is topped by *Hsp70* with a more than hundredfold increase in expression compared to RT. Besides molecular chaperones, notable examples of the utmost upregulated genes are *Frost*, which has been identified for being one of only few known genes that respond strongly towards a cold- but not a heat-shock [Goto, 2001], and a couple of genes involved in immunity (see also Discussion), e.g. *Jun-related antigen* [Kim et al., 2007], *Drosomycin-like 2* [Tian et al., 2008], and *Cecropin C* [Hoffmann et al., 1993]. Overall, the upregulated genes are enriched for a wide variety of often broad GO terms,

including regulation, localization, response to stimulus, immune response, (protein) binding, plasma membrane, cytoplasm, cell cortex and junction, cytoskeleton and ESCRT complex. The downregulated genes, on the other hand, are enriched for e.g. oxidation-reduction, lipid metabolism and intracellular membrane bounded organelle. Interestingly, four of the 16 genes with a log2 fold-change (L2FC) significantly below -1 contain a major facilitator transmembrane transport domain. Another three of these 16 genes have a poorly characterized domain of unknown function (DUF-227), which based on sequence similarity might be involved in the deactivation of ecdysteroid growth hormones.

General expression differences between populations

In order to identify general characteristics of differentiation between the continents, the numbers of mapped reads for each gene were compared between European and African samples across all experimental conditions. 3486 genes show a significantly higher expression in Europe ([von Heckel et al., 2016] Table S8) and 3440 genes a significantly higher expression in Africa ([von Heckel et al., 2016] Table S9), meaning that almost 50% of all genes are differentially regulated due to continental origin. The heavily Europe-biased genes include well known examples as *Cyp6g1* [Daborn et al., 2002] and *Cyp6g2* [Daborn et al., 2007], which are involved in insecticide resistance. *Cyp6g1* is consistently about fourfold upregulated in Europe in the present study and in two other transcriptomic studies [Hutter et al., 2008; Müller et al., 2011], in both of which it is the gene with the strongest overexpression in

Europe. Overall, the Europe-biased genes are GO enriched most prominently for terms related to protein biosynthesis. A GO analysis of the Africa-biased genes, in turn, reveals, for instance, an overrepresentation of genes that play a part in development, regulation, binding, and/or belong to the nucleus.

Genotype by environment interactions

Overall, the transcriptional cold shock response is fairly similar in Europe and Africa. There is not a single gene that is at the same time significantly upregulated due to the cold shock in one population and downregulated in the other. Furthermore, almost all genes that respond strongly towards the cold shock do so in a similar fashion in both populations. Looking for a statistical interaction between the effects of origin and condition, 16 such genotype-environment-interaction (GEI) genes (Table 3) were identified. Two of those, namely *HR38* and *CG44247*, display GEI for rec15 vs. RT. In both cases expression first decreases at rec15 in the European population before it increases at rec90. In the African population, on the contrary, expression gradually increases after the cold shock. At RT and rec90 the two genes are similarly expressed in both populations. The other 14 GEI genes exhibit a population specific regulation for rec90 vs. RT. For six of these genes the L2FC is smaller in Africa and for eight genes larger. Interestingly, for 14 and 15 of the 16 GEI genes the absolute extent of the cold-induced change in expression is greater in the African population at rec15 and rec90, respectively (Table 3). There is only a single gene (*cwo*) for which the absolute change in mRNA abundance is substantially larger in Europe.

Table 3 | Genes with significant genotype by environment interaction

Gene	Significance of interaction FDR(BH)	L2FC rec15 vs. RT Europe	L2FC rec15 vs. RT Africa	L2FC rec90 vs. RT Europe	L2FC rec90 vs. RT Africa
HR38	4.04E-02[a]	**-0.21**	**0.59**	**3.10**	**3.24**
CG44247	4.05E-02[a]	-0.26	0.24	**0.41**	**0.55**
GATAe	9.71E-04	**-0.11**	**-0.61**	**-0.36**	**-1.63**
CG13607	1.06E-03	**-0.40**	**-0.72**	**-0.83**	**-2.67**
CG11897	1.09E-03	**-0.06**	**-0.35**	**-0.10**	**-0.69**
CG11741	1.99E-02	**-0.74**	**-1.03**	**-0.62**	**-1.86**
cwo	1.41E-02	**-0.17**	**-0.44**	0.81	0.04
rudimentary	1.63E-02	**0.07**	**-0.31**	**0.08**	**-0.44**
wunen	7.82E-06	**0.04**	**0.18**	**0.49**	**1.14**
brummer	1.33E-04	-0.19	0.15	**0.39**	**1.26**
CG18744	4.51E-04	**0.43**	**1.29**	**0.93**	**2.29**
CG7017	1.73E-03	**-0.25**	**0.41**	**0.93**	**3.75**
Lnk	3.24E-03	**-0.04**	**0.12**	**0.14**	**0.69**
CG15126	1.12E-02	**0.17**	**0.48**	**0.86**	**1.66**
CG13482	1.99E-02	**0.70**	**1.25**	**1.22**	**2.55**
GstE8	4.96E-02	**0.11**	**0.50**	**0.49**	**1.42**

Significance of interaction applies to rec90 vs. RT except noted otherwise
[a]Interaction is significant for rec15 vs. RT
Bold L2FC values indicate greater absolute L2FC for the African population
Published in [von Heckel et al., 2016].

Genome-wide canalization of European gene expression

To further explore this pattern on a genome-wide scale, the cold-induced L2FC in expression (rec90 vs. RT) was computed for all genes with sufficient read count in both populations (n = 13803 genes) separately for African and European flies using DESeq2 [Love et al., 2014]. Genes were then grouped into distinct bins with a width of 0.2 according to their L2FC (Figure 20). Bins range from extreme downregulation to no change in expression to strong upregulation. The majority of genes exhibit only minor changes in gene expression and the amount of up- and down-regulated genes is comparable, resulting in an approximately normally distributed histogram in both

populations. In the overlay of the histograms for the European and the African population, however, one can see that in the European population bin size is relative to the African population larger for bins with a small absolute L2FC and smaller for bins with a high absolute L2FC (Figure 20). In other words, more genes show a particularly strong cold-induced up- or downregulation in the African population. Thus, gene expression is canalized across control and post cold shock conditions in Europe.

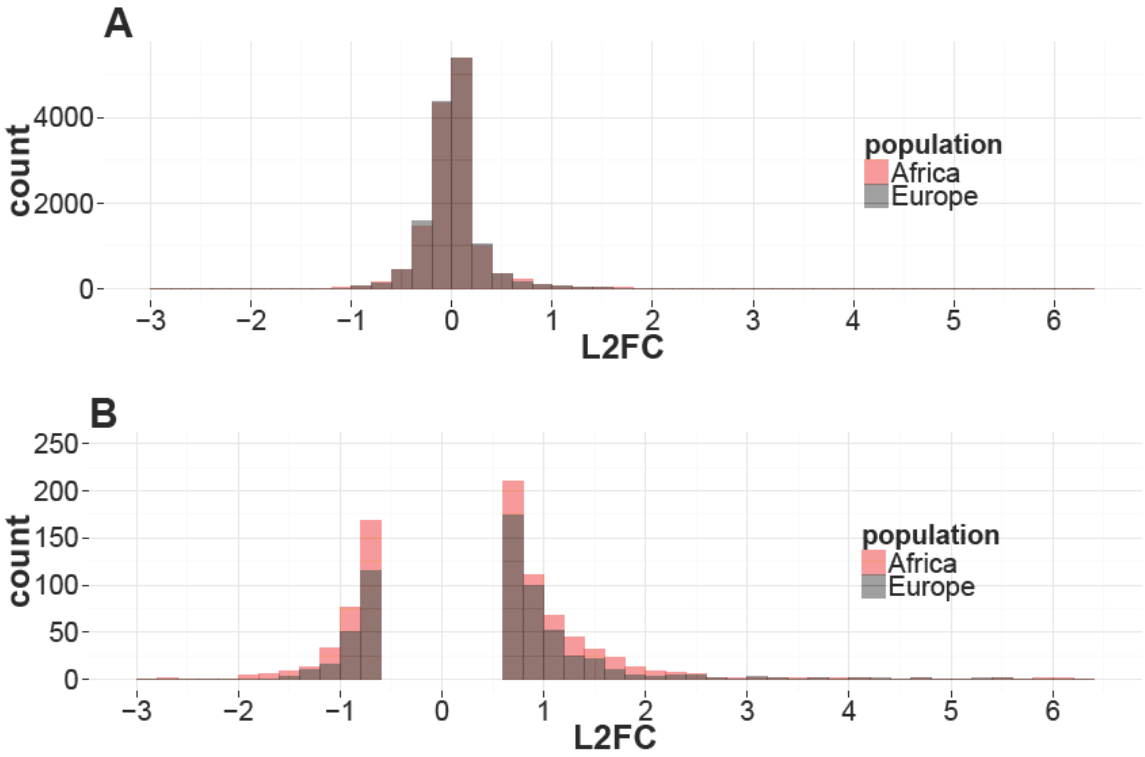

Figure 20 | Genome-wide L2FC per population
Using DESeq2 [Love et al., 2014] the log2 fold-change (L2FC) in expression between rec90 and RT was calculated for 13803 genes with sufficient read count in both populations separately for African and European flies. Genes were then grouped into distinct bins according to their L2FC. Bin width is 0.2. The area where the African and European histograms overlap is depicted in dark red.
(A) All genes, (B) genes with an absolute L2FC > 0.6
Published in [von Heckel et al., 2016].

To corroborate these findings, this pattern was additionally verified in the individual strains. In this case, the genewise L2FC for each strain was calculated after normalization of read counts by the TPM method [Li & Dewey, 2011; Wagner et al., 2012] for all 12617 genes with at least one read in every sample. In all four African strains the amount of genes with a high absolute L2FC between rec90 and RT exceeds the numbers in all four European strains (Figure 21). For the expression change between rec15 and RT this pattern is much less clear. Still, numbers of genes with an absolute L2FC > 1 are on average higher for the African strains.

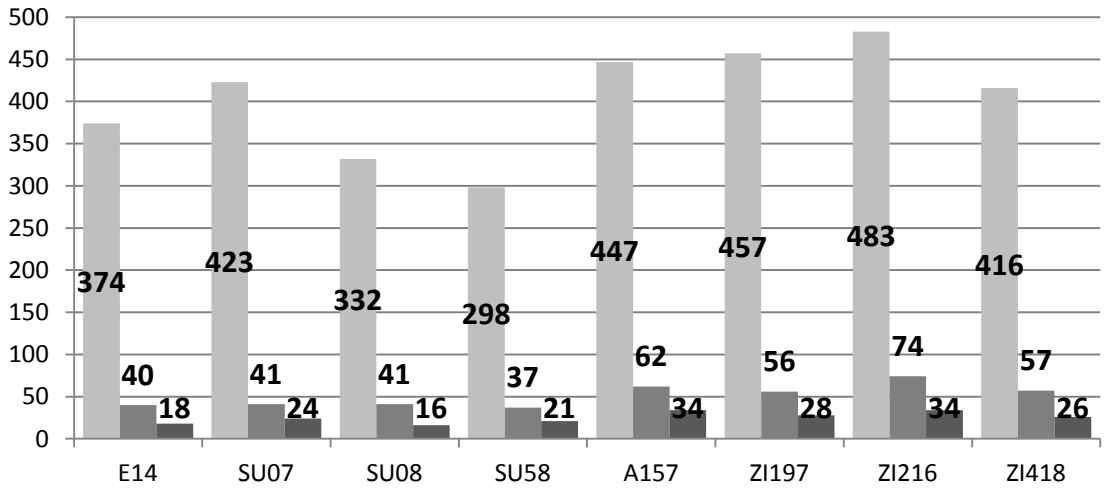

Figure 21 | Amount of highly plastic genes per strain rec90 vs. RT
The y-axis shows the number of genes that fall into the respective bins. The first column per strain shows the amount of genes with an absolute log2 fold-change (L2FC) between 1.0 and 2.0, the 2nd column genes with an L2FC between 2.0 and 3.0, and the 3rd genes with an L2FC above 3.0. Genewise L2FC was calculated for each strain after normalization of raw counts using the TPM method [Li & Dewey, 2011; Wagner et al., 2012]. All genes with zero read count in one sample were excluded resulting in 12617 genes in total. Depicted are only genes with an absolute L2FC > 1 with no respect to up- or down-regulation. Modified from [von Heckel et al., 2016].

2.7 Functional testing of candidate genes

It has been previously reported that an RNAi induced single gene knockdown of *Frost*, *Hsp22*, and *Hsp23* measurably diminishes cold tolerance [Colinet et al., 2010a; Colinet et al., 2010b]. However, an effort to replicate these results for *Frost* was unsuccessful [Udaka et al., 2013]. Following the reported experimental approach, 47 candidate genes for cold tolerance, including the three aforementioned, were knocked down in expression using the VDRC RNAi library [Dietzl et al., 2007] and two ubiquitous driver lines (α-Tubulin and Act5C). To this end, virgin females of the driver lines were crossed to young males of the respective IR lines from the VDRC RNAi library. Since the driver lines are not homozygous, F1 progeny was screened using phenotypic markers to obtain the desired knockdown cross. Efficiency of the knockdown was determined via qPCR for the example of the gene *Frost*. Finally, the consequences of the individual gene knockdowns on cold tolerance were assessed in comparison to the appropriate control crosses. The experimental approach was directed at identifying genes, for which the knockdown results in strong effects on cold tolerance that are in the range of the effects that have been reported for *Frost* by Colinet et al. [Colinet et al., 2010a]. The rationale was to perform an initial screen for a relatively large number of single gene knockdowns at various experimental conditions and, if some knockdowns show promising results to then repeat the experiment for these knockdowns at the proper conditions. This would allow for extensive testing of the respective gene(s) in the follow up phase, the quantification of the particular knockdown efficiency and, additionally, would minimize multiple testing. Because it was

not possible to test all knockdowns at all conditions and with both driver lines due to the enormous experimental effort, they were mostly assigned to a particular group that was tested only at one distinct experimental condition and with only one driver line. As a result, the initial screen is somewhat unsystematic.

Viability of individual gene knockdowns

Twenty-six single gene knockdowns yielded viable offspring without any apparent morphological abnormalities in at least one of the two crosses. In three of these cases (*Hsp26*, *CG31689* and *brinker*) the knockdown was viable only when using the αTub driver. For one gene (*Hsp68*) the knockdown was viable only in female flies. Thus, for 22 genes the knockdown was viable with either driver line and in both sexes, whereas the knockdown was completely lethal for 18 genes. For three genes (*Hsp27*, *CG11897* and *CG13321*) the knockdown yielded only few adult flies, which in most cases also had severely malformed wings and strongly reduced longevity. An impaired cold tolerance in these flies is likely the result of a general reduction in vigor and the genes, therefore, cannot be specifically linked to cold tolerance.

An overview of the viability of the knockdown for all 47 tested genes is shown in Table 4.

Table 4 | Viability after a ubiquitous gene knockdown

VDRC_ID	CG number	Gene name	Viability
100095KK	CG10449	*Catsup*	not viable
100619KK	CG5670	*Atpα*	not viable
100955KK	CG4183	*Hsp26*	viable/not viable*
101019KK	CG13762	*brivido-3*	viable
101174KK	CG10369	*Irk3*	viable
101669KK	CG4466	*Hsp27*	low, malformed
101887KK	CG9653	*brinker*	viable/not viable*
102049KK	CG9434	*Frost*	viable
102097KK	CG31689	*CG31689*	viable/not viable*
102270KK	CG7084	*CG7084*	not viable
102426KK	CG9568	*CG9568*	not viable
102403KK	CG12164	*CG12164*	viable
102493KK	CG4463	*Hsp23*	viable
103009KK	CG13510	*CG13510*	viable
103377KK	CG7390	*smp-30*	not viable
103646KK	CG17367	*Lnk*	viable
103666KK	CG5925	*desat2*	not viable
103919KK	CG5232	*sas*	viable
104178KK	CG1864	*HR38*	not viable
104618KK	CG10578	*DnaJlike*	not viable
104883KK	CG1615	*Ork1*	not viable
105174KK	CG11897	*CG11897*	low, malformed
105442KK	CG8778	*CG8778*	viable
105510KK	CG13321	*CG13321*	low, malformed
105551KK	CG10912	*CG10912*	not viable
105596KK	CG43690	*fok*	viable
105655KK	CG5290	*CG5290*	not viable
105893KK	CG34413	*NKAIN*	not viable
106548KK	CG31509	*TotA*	viable
106655KK	CG33135	*KCNQ*	viable
106787KK	CG17752	*CG17752*	viable
107356KK	CG5436	*Hsp68*	♀viable/♂not viable
107389KK	CG6747	*Irk1*	viable
108269KK	CG15678	*pirk*	viable
108300KK	CG3152	*Trap1*	viable
108568KK	CG1242	*Hsp83*	not viable
108683KK	CG3478	*pickpocket*	not viable
109475KK	CG5953	*CG5953*	viable
110058KK	CG16700	*CG16700*	not viable
03245GD	CG10913	*Serpin55B*	not viable
06198GD	CG14205	*CG14205*	viable
28480GD	CG34329	*Diedel3*	viable
34408GD	CG32130	*starvin*	not viable
36640GD	more than 1	*Hsp70*	viable
41748GD	more than 1	*Hsp70*	not viable
43632GD	CG4460	*Hsp22*	viable
44395GD	CG7130	*CG7130*	viable

*knockdown was viable with the αTub driver but lethal with Act5

Efficiency of the gene knockdown in expression

The RNAi induced knockdown of *Frost* was verified via qPCR. Female and male flies of the knockdown cross using the α-Tubulin driver and the respective control were tested 90 minutes after a 7h cold shock. For the Act5C driver expression was analyzed 90 minutes after an 18h cold shock. According to expectations, expression of *Frost* is greatly (more than tenfold) reduced in the knockdown compared to the control with both driver lines and for both sexes (Figures 22&23). The knockdown efficiency is in line with similar experiments that have been carried out for other genes using the same framework (Amanda Glaser-Schmitt, personal communication) and with the results reported by Colinet et al. [Colinet et al. 2010a; Colinet et al. 2010b].

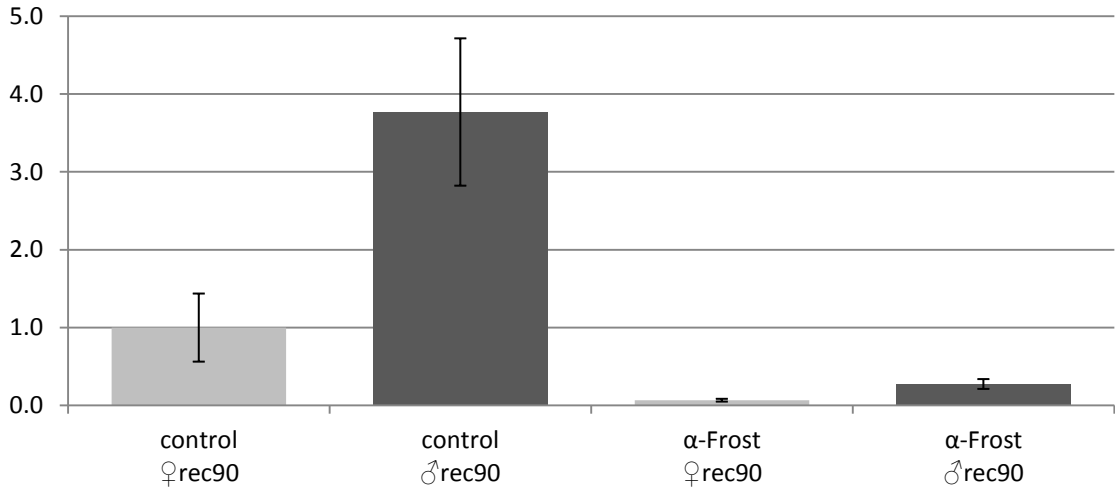

Figure 22 | Efficiency of the *Frost* knockdown using the α-Tubulin driver
The y-axis shows expression relative to the first column (female control), which is set to one. Samples were taken 90 minutes after a 7h cold shock for female and male flies of the control and the *Frost* knockdown (α-Frost). Expression values are calculated as the geometric mean of four biological replicates. In the knockdown expression is reduced to 6.7% for females and to 7.3% for males compared to the respective control. Error bars denote the standard deviation.

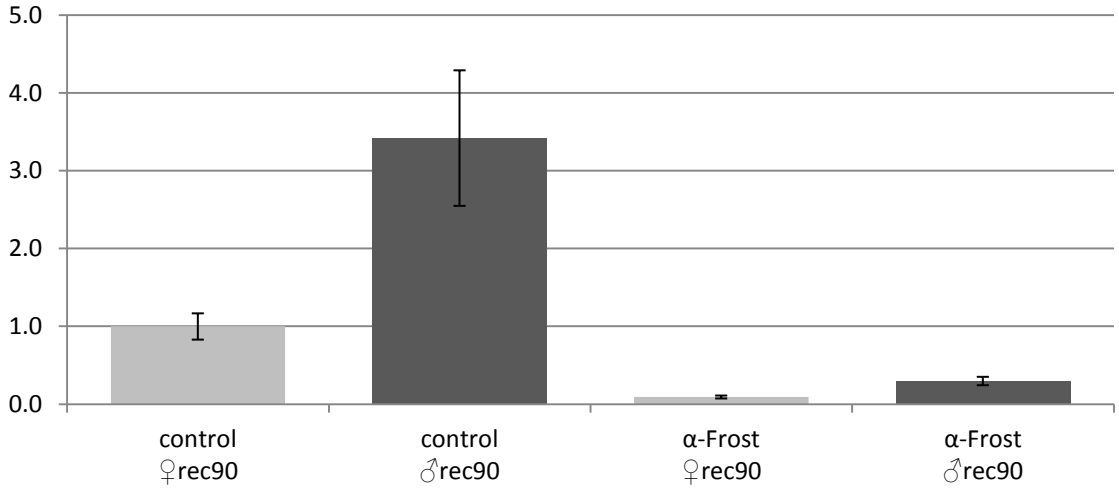

Figure 23 | Efficiency of the *Frost* knockdown using the Act5C driver
The y-axis shows expression relative to the first column (female control), which is set to one. Samples were taken 90 minutes after an 18h cold shock for female and male flies of the control and the *Frost* knockdown (α-Frost). Expression values are calculated as the geometric mean of four biological replicates. In the knockdown expression is reduced to 9.4% for females and to 8.7% for males compared to the respective control. Error bars denote the standard deviation.

Cold tolerance phenotypes after knockdown of individual genes

Twenty-six single gene knockdowns were functionally tested for an effect on CCRT and oftentimes as well for an effect on 24h cold shock survival. An overview of all tested gene knockdowns including the particular experimental conditions, the obtained CCRT and survival values, and the significance of the difference of these values to their respective controls' values with and without multiple testing correction can be found in the Appendix (Tables A1-A10). Most notably, neither for *Frost*, nor for *Hsp22*, nor for *Hsp23* does the knockdown increase CCRT or decrease cold shock survival (Figures 24-27, see also Appendix, Tables A1-A10) in a fashion comparable to what had been originally reported [Colinet et al. 2010a; Colinet et al. 2010b].

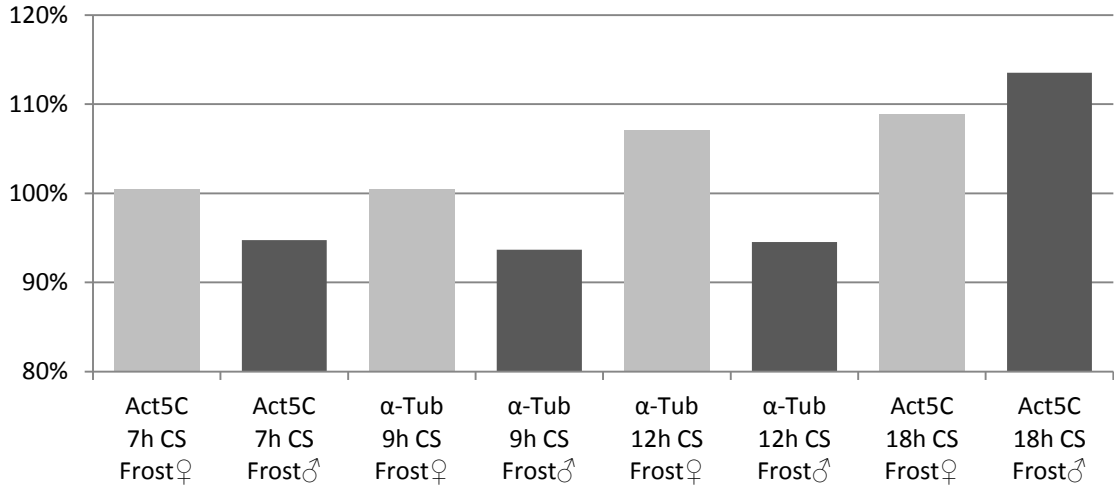

Figure 24 | CCRT after ubiquitous *Frost* KD relative to control
The y-axis shows CCRT values relative to the respective control with the same driver line, cold shock duration and sex. Underlying data is detailed in the Appendix (Tables A1-A10). For comparison: [Colinet et al., 2010a] report a strong increase in CCRT for female and male flies after a 12h CS using the α-Tubulin driver.

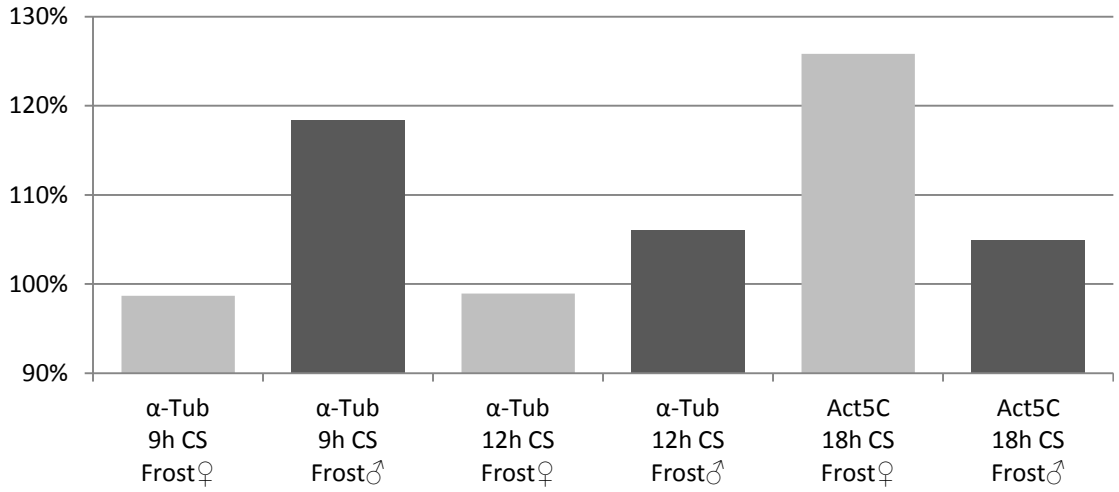

Figure 25 | 24h survival after ubiquitous *Frost* KD relative to control
The y-axis shows 24h survival values relative to the respective control with the same driver line, cold shock duration and sex. Underlying data is detailed in the Appendix (Tables A1-A10). For comparison: [Colinet et al., 2010a] report a >50% reduction in survival for female flies and a slight reduction in survival for male flies after a 12h CS using the α-Tubulin driver.

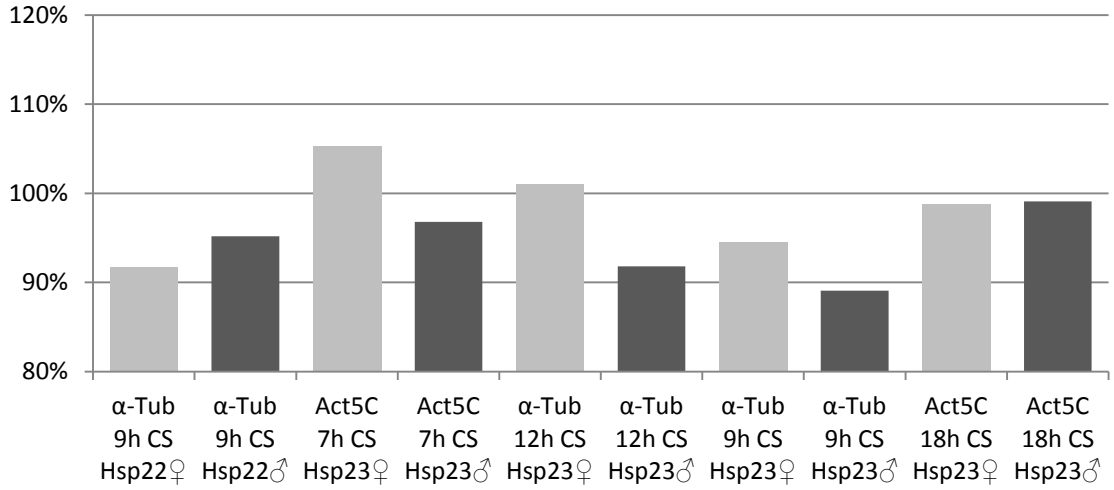

Figure 26 | CCRT after ubiquitous *Hsp22*/*Hsp23* KD relative to control
The y-axis shows CCRT values relative to the respective control with the same driver line, cold shock duration and sex. Underlying data is detailed in the Appendix (Tables A1-A10). For comparison: [Colinet et al., 2010b] report a substantial increase in CCRT for female and male flies after a 12h CS using the Act5C driver.

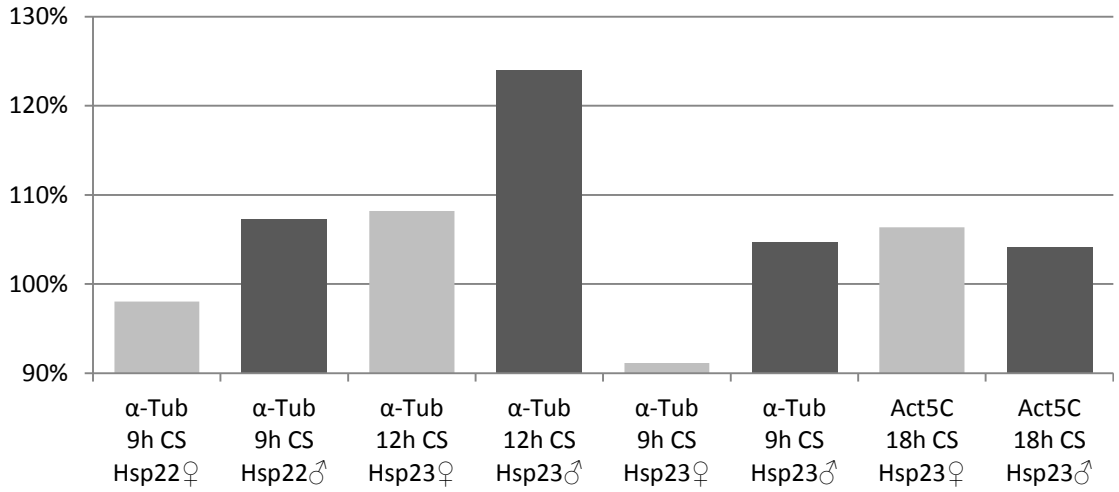

Figure 27 | 24h survival after ubiquitous *Hsp22*/*Hsp23* KD relative to control
The y-axis shows 24h survival values relative to the respective control with the same driver line, cold shock duration and sex. Underlying data is detailed in the Appendix (Tables A1-A10). For comparison: [Colinet et al., 2010b] report a marginal decrease in survival for female and male flies after a 12h CS using the Act5C driver.

Likewise, for all other tested genes the consequences of the gene knockdowns are marginal. When multiple testing correction is applied, the only cases of a significantly impaired cold tolerance are increased CCRT values for the knockdown of *CG12164* and *TotA* with the Act5C driver after an 18h CS (Table A9). However, these observations do not correspond to a decrease in cold shock survival and apply only to one sex. Additionally, the assessment of CCRT after such a long cold shock is somewhat imprecise (see Discussion, Chapter 3.3).

3. Discussion

3.1 Cold tolerance phenotypes in tropical and temperate populations of *Drosophila melanogaster*

The populations from Siavonga, Zambia (ZI) and Umea, Sweden (SU) are particularly suitable for the subsequent examination of the genetic basis of cold adaptation. The Siavonga population (ZI) from Zambia stems from a truly tropical environment and shows the highest genetic variation that has been observed for any *D. melanogaster* population to date [Pool et al., 2012]. Thus, this population is potentially close to the source of all extant cosmopolitan *D. melanogaster* populations. The Swedish population from Umea (SU), on the other hand, constitutes a derived case from the northernmost tip of the species range [Wollstein et al., unpublished]. Accordingly, CCRT of Zambian flies is consistently and considerably longer than for Swedish flies. While there are huge variations within populations, this pattern holds on average with a mean CCRT of 41.1 - 42.8 minutes (95% CI, n=437; every n represents the average CCRT of ~10 flies) for ZI and 28.3 - 29.2 minutes (95% CI, n=484) for SU. Notably, these population differences in CCRT persist over a wide range of environmental conditions. Within the respective populations SU07, SU08 and SU58 are the fastest and ZI197, ZI216 and ZI418 the slowest recovering strains whose complete genome sequences are readily available. The Dutch strain E14 has the shortest CCRT of all strains investigated, while the Zimbabwean strain A157 displays a very long CCRT, which is in the range of the slowest Zambian strains. Thus, it was chosen to use these eight strains for

the genome-wide analysis of regulatory differences related to cold tolerance.

3.2 The transcriptional response towards a cold shock in tropical and temperate populations of *Drosophila melanogaster*

The genome-wide transcriptional response towards a cold shock was measured in temperate European and tropical African populations of *Drosophila melanogaster* via RNA sequencing. The data shows, first of all, that there is very little change in expression during the cold shock, which is probably due to a general halt of transcription at 0°C. Secondly, already 15 minutes after the end of the cold shock several hundred genes are differentially expressed compared to RT and after 90 minutes of recovery this number further increases to encompass roughly a third of all genes. Many of the genes that are most strongly upregulated belong to the heat shock protein family or to other classes of molecular chaperones. Thirdly, despite ample population differences in baseline expression, the cold shock response appears fairly similar in both populations, with only 16 genes showing a statistical interaction between the effects of origin and condition. It is precisely this kind of genotype-environment interaction (GEI) that is considered to be the hallmark of local regulatory adaptation. Interestingly, the great majority of these GEI-genes exhibit a stronger extent of cold-induced change in the African population. A similar pattern is visible on a genome-wide scale, where numbers of genes with exceedingly sharp up- and downregulation in response to the cold shock are much higher in Africa.

Decreased thermosensitivity of gene expression in temperate flies

Phenotypic variability in nature is constrained. Strikingly uniform phenotypes appear in high frequency in a population despite genetic and environmental variation. The phenomenon that results in this phenotypic robustness has been termed canalization [Waddington, 1942]. Its conception is diverse (see [Flatt, 2005] for a review) and debated in many aspects, e.g. whether or not it is necessarily promoted by natural selection [Siegal & Bergmann, 2002]. Canalization is often invoked with regard to the morphological development of an organism, but likewise applies throughout adult stages (e.g. [Hatle et al., 2003]) and to more subtle phenotypic features like gene expression [Manu et al., 2009; Shaw et al., 2014; Chanderbali et al., 2010].

Ninety minutes after the cold shock (and to a lesser extent also 15 minutes after the cold shock) the genome-wide cold-induced change in expression is considerably smaller in the cold-tolerant European population. While the direction of transcriptional change for a given gene, i.e. up- or downregulation, is mostly the same in the two populations, the extent of the cold-induced change is often larger in the cold-sensitive African population (Figure 20). This pattern of canalization of gene expression in temperate flies has been observed in other studies in *D. melanogaster* [Levine et al., 2011; Voigt et al., 2015; Zhao et al., 2015] and there is evidence that it is under positive selection [Voigt et al., 2015]. On the other hand, extreme cold sensitivity has been associated with an exaggerated transcriptional stress response [Vermeulen et al., 2013]. Accordingly, exposure to zero degrees, which is a novel stressor for the African flies, might elicit an overshooting stress

response or disturbance of regulatory networks, while European flies have adapted to better maintain certain optimal transcript levels. The rec90 time point was chosen, because in the qPCR experiments (Chapter 2.5) and in the literature [e.g. Colinet et al., 2010a&b&c] some prominent stress response genes peak in expression around this timepoint. This is true for both European and African flies and there is no evidence that this peak in expression generally occurs later in the African population and that thus the canalization pattern at this time point is solely the result of a timing shift. Though even if this would be the case, the faster return towards baseline expression patterns in the European flies might still present a case of canalization and be potentially adaptive. It is, however, difficult to directly relate these regulatory differences to differences in CCRT based on the expression data alone. Most parts of the transcriptional cold shock response are likely not overly relevant for chill coma recovery. Rather, faster chill coma recovery and canalization of gene expression of European flies are both phenotypic characteristics of cold adaptation. It would require additional timepoints in the recovery phase to identify individual genes whose expression directly follows the particular chill coma recovery dynamics in the different populations.

Transcriptional change during the cold shock

Several genes with GO terms related to the (negative) regulation of metabolism/transcription are downregulated during the cold shock. Since *de novo* transcription in *D. melanogaster* is apparently very limited at 0°C as the transcriptomic data suggests (see also [Sinclair et al., 2007; Colinet et al.,

2010c]), this pattern might be caused by active and specific RNA degradation, which remains possible at very low temperatures [Ma et al., 2004]. The downregulation of genes that play a part in regulatory processes in the nucleus may represent a preparation for the burst of gene expression in recovery phase. However, due to the small change in expression of all of these genes, further work is required to confirm these findings, possibly after a prolonged cold shock to allow for the accumulation of slow changes.

Chaperones, the cytoskeleton and the stabilization of membranes

The cold shock response in the recovery phase is characterized by the massive upregulation of molecular chaperones. These proteins bind to other proteins and are responsible for the reversion of undesirable conformational changes induced by stressors and assist in the degradation of irreversibly misfolded proteins [Kriegenburg et al., 2012]. Several of these chaperones, most prominently members of the heat shock protein family, have previously been shown to be strongly upregulated after a cold shock in *Drosophila* [Colinet et al., 2010c; Qin et al., 2005] and other insect species [Teets et al., 2012; Dennis et al., 2015]. The chaperonin-containing T-complex (CCT) is a ring-shaped complex, which consists of eight different subunits and is involved in the folding of nascent cytoskeletal proteins [Sternlicht et al., 1993]. The cytoskeleton plays an important role in cold hardening in plants [Orvar et al., 2000] and cytoskeletal genes have been shown to be upregulated in several insects after cold exposure [Kim et al., 2006; Colinet et al., 2007; Teets et al., 2012]. In pupae of the onion fly *Delia antiqua* high mRNA levels of CCT genes

correlate with cold hardiness and prevent actin-depolymerization, thus stabilizing the membrane [Kayukawa & Ishikawa, 2009]. All eight subunits of the CCT are strongly upregulated at rec90. Additionally, there is an upregulation of the major component of the actin cytoskeleton *Actin5C*, of all eight subunits of the Arp2/3-complex, which is responsible for Actin-polymerization and branching, and of *rhea*, which is responsible for the anchoring of the cytoskeleton in the plasma membrane. Altogether, this has the potential to strengthen the cytoskeleton and its connection to the membrane and hence to increase membrane stability and to prevent extensive ion leakage. Besides, the actin cytoskeleton takes part in intracellular protein transport [Stamnes, 2002], which might be in particular demand after the cold shock to get rid of denatured proteins and to provide for repair and structural adjustments. The importance of protein degradation is further corroborated by an upregulation of the endosomal sorting complex required for transport (ESCRT) [Vaccari et al., 2009], which is GO enriched among the upregulated genes at rec90. Again, processes related to vesicular transport have been found to be upregulated in response to a cold shock in other insects as well [Teets et al., 2012].

The expression of immune genes in response to the cold shock

In *D. melanogaster* the response towards a cold shock has been associated with an increased expression of immune related genes [Zhang et al., 2011; Vermeulen et al., 2013] and there are various hypotheses why this might be the case [Marshall & Sinclair, 2012]. In the present study the 120 genes that

are consistently more than twofold upregulated at rec90 show a significant enrichment of the GO term “response to biotic stimulus”. The 11 genes that drive this pattern are *Hsp27*, *Relish*, *ets at 21c*, *pdgf- and vegf-receptor related*, *poor imd response upon knock-in*, *Drosomycin-like 2*, *unc-45*, *daughter of sevenless*, *unpaired 3*, *pancreatic eif-2alpha kinase*, and *Cecropin C*. Likewise, several immune related terms are GO enriched among all upregulated genes at rec90. The upregulation of immune related genes is on average marginally stronger in the African population, but it is often very inconsistent among strains and even among biological replicates. The flies that were used for the generation of the transcriptomic data were healthy in appearance, but they were grown under standard lab conditions and not in a sterile environment, so it is not possible to rule out differences in microbial load and other factors that might influence the immune system. Still, the data suggests considerable crosstalk between the cold shock response and the immune system, albeit for several genes in a somewhat erratic fashion.

Conserved patterns of the transcriptional cold shock response

Gene expression studies that try to answer very similar questions using different biological material and/or different methodologies often arrive at vastly different results. Patterns that are conserved despite of these minor experimental differences, are likely more reliable and relevant in nature. Here, the results of the present study are compared with few other studies that likewise examine the transcriptional cold shock response using different fly populations, experimental approaches and means of quantifying gene

expression [Qin et al., 2005; Sinclair et al., 2007; Colinet et al., 2010c; Zhang et al., 2011]. Qin et al. [Qin et al., 2005] measured the change in gene expression 30 minutes after a 2h cold shock via microarrays in 5-7d old males. They identify 31 upregulated genes, grouped into five functional categories: stress response, membrane, mitochondrial and energy, expression, and other. Whereas all five stress response genes (*Hsp83*, *Hsp 26*, *Hsp23*, *Ubiquitin-63E*, and *Frost*) are also upregulated in the present study at rec90, only 11 of the remaining 26 genes including *CG10912* show this pattern as well. Of the six downregulated genes [Qin et al., 2005], three show a downregulation at rec90 including *smp-30*. Sinclair et al. [Sinclair et al., 2007] monitored expression of five genes, namely *Frost*, *smp-30*, *Hsp23*, *Hsp70* and *Desat2* during a 3h cold shock and in the subsequent 3h recovery period in 5d old males. They see no change in expression during the cold shock, but an upregulation for *Frost* and *Hsp70* and a downregulation for *smp-30* in the recovery phase. In contrast to the present study, they observe no upregulation of *Hsp23* and a slight increase in the expression of *Desat2*, which is strongly downregulated after the cold shock in our study. In agreement with Colinet et al. [Colinet et al., 2010c], who subjected 4d old virgin male flies to a 9h cold shock, all Hsp are strongly upregulated in response to the cold shock, with the exception of *Hsp60* and *Hsp67*, which consistently show only relatively weak upregulation. Zhang et al. [Zhang et al., 2011] subjected virgin females to three different cold treatments with short (2h CS), prolonged (10h CS) and repeated (2h CS on five consecutive days) exposures to cold, which results in largely non-overlapping gene expression

changes 6h after recovery. They find only three genes (*TotA*, *hephaestus*, *CG11374*) to be upregulated in all three treatments. Of these *hephaestus* is the only gene to be upregulated in the present study, albeit only to a marginal extent (L2FC rec90 vs. RT = 0.10) and with a FDR slightly above 5% (0.079). Expression of *CG11374* remains relatively constant over the different conditions, whereas the expression of *TotA* is characterized by huge variations between biological replicates and, thus, likely influenced by factors other than the cold shock. The same is true for the other members of this gene family (*TotC*, *TotM*) that were also upregulated in at least one of the cold treatments in the study by Zhang et al. Of the 20 genes that are differentially regulated in two cold treatments in their study, six are likewise affected at rec90. *CG15043*, *Attacin A*, *urate oxidase*, and *Attacin B* are consistently upregulated, whereas *CG9463* and *CG15533* are strongly downregulated.

Conserved regulatory population differences

Additionally, baseline differences in gene expression are compared with three studies that likewise assess regulatory differences between African and European populations in whole male [Hutter et al., 2008; Paparazzo et al. 2015] and female [Müller et al., 2011] flies. Performing differential expression analysis on the RT samples only, i.e. on unstressed flies for a dataset encompassing eight samples per population, 867 genes display Europe-biased and 793 genes Africa-biased expression at a 5 % FDR cutoff. The only study that uses an identical technical framework, i.e. RNAseq plus the same mapping procedure and genome annotation, is Paparazzo et al. [Paparazzo et

al. 2015]. They have performed RNAseq in threefold biological replication for mass bred Dutch and Zimbabwean populations that were generated by outcrossing 12 Dutch (including E14) and 10 Zimbabwean (including A157) inbred lines. Their baseline controls, however, may technically not have been completely unstressed, since stripes with oil were inserted into the fly vials (see [Paparazzo et al. 2015] for details). For these six samples 206 Europe-biased and 322 Africa-biased genes are identified. Only 27 (13.1 %) and 25 (7.8 %) genes are significantly overexpressed in Europe and Africa, respectively, in both the present study and their dataset. The 27 shared Europe-biased genes include *Cyp6g1*, *Cyp6g2*, *Cyp6t3*, and *Cyp313a1*, all of which belong to the Cytochrome P450 gene family and are implicated in the response to insecticides. An example of the shared Africa-biased genes is *Amyrel*. Furthermore, 17 genes are differentially expressed in an opposite fashion in the two datasets. The comparison between the present study and Hutter et al. [Hutter et al., 2008] and Müller et al. [Müller et al., 2011] is hampered by technical differences, since these two studies use microarrays and different genome annotations. Thus, datasets that only include genes that are represented in each of the respective studies were generated. For the comparison with Hutter et al. [Hutter et al., 2008] this dataset encompasses 4,500 genes, for the comparison with Müller et al. [Müller et al., 2011] 5,216 genes, and for a comparison between all three datasets 2,354 genes. Both studies compare gene expression differences between several (8-12) Dutch and Zimbabwean inbred lines. For Hutter et al. [Hutter et al., 2008], eight of the 74 genes (10.8%) overexpressed in Europe overlap with the present

study, including *Cyp6g1*, *CG9509*, and *Malic enzyme*. Twenty-three of 85 genes (27.1%) are, on the other hand, consistently upregulated in Africa. Interestingly, the sole gene with an opposing expression pattern is *CG10912*, which is strongly upregulated in response to the cold shock in the present study and other studies [Qin et al., 2005; Zhang et al., 2011]. This gene is Africa-biased in [Hutter et al., 2008] and Europe-biased in the present study. For Müller et al. [Müller et al., 2011], 19 of 312 genes (6.1%) are Europe-biased in the present study as well and 15 of 244 genes (6.1%) are Africa-biased, whereas 31 genes show an opposing pattern. The smaller proportion of overlapping genes and the higher amount of genes that display differential expression in opposite directions when comparing the present study to Müller et al. [Müller et al., 2011] relative to comparing it with Hutter et al. [Hutter et al., 2008] is likely owed to sex-specific differences in gene expression. *Cyp6g1* is the only gene that is overexpressed in Europe in both of these studies and the present study, whereas two genes, namely *Actin 88F* and *retinin*, are consistently Africa-biased. If the study by Paparazzo et al. [Paparazzo et al., 2015] is included, *Cyp6g1* and *retinin* are the sole genes to be overexpressed in Europe and Africa, respectively, in all four studies. While expression of *Cyp6g1*, which is also overexpressed in European *D. simulans* [Wurmser et al., 2013], is associated with DTT resistance [Daborn et al., 2002], *retinin* is a cornea-specific protein and likely secreted into extracellular space [Kim et al., 2008]. Overall, the amount of genes that show a consistent pattern with regard to regulation differences between Europe and Africa is considerably small.

Limitations and perspectives

Though the genome-wide transcriptional response towards a cold shock was analyzed at three different timepoints and in natural fly populations of vastly differing cold tolerance in extensive replication, the present study remains limited with respect to several aspects. First, only male flies were employed to avoid the impact of pregnancy while at the same time not being forced to separate virgin females early on. There are, however, huge regulatory differences between the sexes [Ellegren & Parsch, 2007; Müller et al., 2012]. Thus, results from male flies cannot easily be generalized. Additionally, the topic of reproductive diapause [Saunders et al., 1989], which is considered to be crucial for overwintering and population persistence in temperate environments, is not covered. Secondly, RNA was extracted from whole flies since there is no conclusive indication for a particular tissue to be of primary importance in the cold shock response and since the cold shock should in principle affect the whole fly. Nevertheless, the cold shock response might vary greatly between tissues, even in opposite directions, and this might partly obscure the obtained results. Thirdly, the transcriptional response towards a single 7h cold shock was measured without any acclimation pretreatments. Different cold shock durations and repeated cold shocks with intermittent recovery periods, however, elicit varied gene expression changes [Zhang et al., 2011]. Moreover, cold rearing and preceding exposures to cold greatly alter cold tolerance related phenotypes (Figure 3) [Kelty & Lee, 1999; Ayrinhac et al., 2004] and patterns of gene expression [Levine et al., 2011; Chen et al., 2015]. Hence, in order to obtain a more comprehensive picture of

the cold shock response and of regulatory population differences that form the basis of cold adaptation, it is necessary to evaluate the cold-induced changes in gene expression in both sexes, different life stages, and for individual tissues over an extensive set of preferably natural conditions. Finally, a common challenge that stress-related transcriptomic studies on sensitive and tolerant organisms face is the difficulty to discern if deviating patterns of stress-induced gene expression in the tolerant organism are in itself adaptive or simply the result of reduced intrinsic stress. This distinction is particularly complicated since functional evidence is often hard to come by.

3.3 Functional consequences of reduced transcript levels of candidate genes for cold tolerance

It has in general been very challenging and often unsuccessful to link differences in gene expression with phenotypic changes. Here, the functional consequences of a single gene knockdown on cold tolerance were evaluated for 26 individual genes, for which the knockdown was viable. The approach was aimed at detecting relatively large and general effects. Although the knockdown reduces expression more than tenfold, it elicits no clear-cut and general phenotypic effect on cold tolerance for any of the 26 genes, though of course this is by no means a proof that there is no such effect.

CCRT and other cold tolerance related traits are highly plastic, intrinsically variable and presumably multigenic. Therefore, the effect size of a single gene knockdown will often be very small and may vary due to minor environmental fluctuations and/or in a random fashion. While the chill coma recovery assay

in the present setup (7h cold shock, experimental procedure according to [Svetec et al., 2011]) is suitable to distinguish temperate and tropical populations, it is associated with a variety of problems that may interfere with the determination of small differences in CCRT: Flies are monitored only in five minute intervals. Sometimes flies get attached to the experimental vials because of condensed water or excretions, which means that they cannot stand up despite being clearly awake. It is not uncommon to observe flies even tearing off their own wings when they are stuck to the plastic vial. Recovered flies may run around in the vials, thereby waking up other flies. Many of these issues can be solved by putting single flies into individual vials and by decreasing observation intervals. This procedure, however, would greatly increase the experimental effort and, thus, is only suitable for the testing of few strains/knockdown crosses. With increasing duration of the cold shock, additional problems arise: flies often do not stand up in an instance, as they usually do after a 7h cold shock, but maneuver themselves in an upright position over the course of a longer period of time, which makes the calling of the exact CCRT somewhat arbitrary. Furthermore, it becomes increasingly widespread that flies that have initially recovered, i.e. have already put themselves on their six legs in an upright fashion, then lose this posture again. Possibly, different means to quantify cold tolerance could be used to overcome these problems. Some authors advocate the use of the critical thermal minimum, i.e. the temperature at which coma sets in, over CCRT for the quantification of cold tolerance [Andersen et al., 2015]. This method, however, would require a device that allows for an exact and gradual

decrease of temperature. Another alternative approach is to measure the proportion of female flies still standing after 96h at 4°C [Pool et al., 2016].

Besides the quantification of cold tolerance, an additional point that may interfere with functional validation of candidate genes is that many of the genes investigated in the present study belong to multigene families and, thus, might be at least partially redundant in function. Furthermore, CCRT and cold survival are just proxies for cold tolerance. Changes in gene expression that do not influence these particular traits might very well still be relevant for cold tolerance. The fact that the replication of the previously reported phenotypic consequences of a knockdown of *Frost*, *Hsp22*, and *Hsp23* [Colinet et al., 2010a; Colinet et al., 2010b] failed, despite closely following the outlined experimental procedure, might also be attributed to minor differences in fly culture or a different genomic background due to different driver lines used for the knockdown crosses. In any case, these findings together with those of Udaka et al. [Udaka et al., 2013], who likewise observe no such effect on cold tolerance after knocking down expression of *Frost*, suggest that the reported phenotypic implications of altered transcript levels of these genes are not general. Moreover, the lack of any clear-cut effect of the gene knockdown for all 26 analyzed genes further strengthens the notion of cold tolerance as a multigenic phenotype with presumably minor effect sizes for individual genes.

4. General conclusion

The present study did not reveal major candidate genes for cold tolerance. Rather than identifying individual genes with vast regulatory population differences, relatively subtle population differences over a wide range of genes were detected. The salient pattern, in this respect, is that many more genes respond strongly towards the cold shock in the cold sensitive African population. The ability to maintain favorable expression levels can be key to cope with extreme environmental fluctuations [Shaw et al., 2014], since plastic changes induced by novel environments are often non-adaptive [Ghalambor et al., 2015]. In *D. melanogaster*, gene expression is known to be more canalized in temperate populations [Levine et al., 2011; Voigt et al., 2015; Zhao et al., 2015]. Furthermore, an exaggerated stress response has been associated with extreme cold sensitivity [Vermeulen et al., 2013]. Taken together, these findings highlight the importance of canalization of gene expression for cold adaptation and emphasize its polygenic nature. On the other hand, however, phenotypic plasticity is the major factor influencing cold tolerance (Table 1) [Ayrinhac et al., 2004] and plastic responses to cold conditions, which also involve changes in gene expression [Levine et al., 2011; Chen et al., 2015], appear to be adaptive at large even in tropical populations (Figure 3) [Ayrinhac et al., 2004]. Thus, special consideration should be directed towards the expression patterns that remain relatively conserved over a wide range of environmental conditions in temperate compared to tropical populations.

5. Material and Methods

5.1 Fly populations and culture

The fly populations that were analyzed in this study, consist of a Swedish (Umea, collected in 2012 by R. Wilches and S. Laurent, described in [Wollstein et al., unpublished]), a Danish (Odder, collected in 2010 [Schou et al., 2014]), a Dutch (Leiden, collected in 1999, [Bubliy & Loeschcke, 2002]), a Zambian (Siavonga, collected in 2010 by R. Corbett-Detig, [Pool et al., 2012]), a Rwandan (Gikongoro, collected in 2008 by John Pool [Pool et al., 2012], and a Zimbabwean (Lake Kariba, collected in 1994 by T. Mutangadura, [Glinka et al., 2003]) population. All strains of the respective populations are isofemale and their heterozygosity has been further reduced due to five to ten rounds of full sib inbreeding, with the exception of ZI81, ZI173, ZI212, ZI261, ZI303, ZI395, ZI468 and ZI504, which are not inbred. For the initial phenotyping (=standard condition), flies were cultured in 50ml glass or plastic vials under standard lab conditions at 21°C ± 1°C, 25-50% air humidity and a 14h/10h light/dark cycle on a sugar beet molasses and cornmeal medium containing agar-agar for medium consistency, dry yeast for oviposition promotion and propionic acid and nipagin as preservatives.

5.2 Chill coma recovery assays

To quantify cold tolerance chill coma recovery time (CCRT) was used as metric [David et al., 1998]. Briefly, CCRT is defined as the time it takes for a fly to recover, i.e. to stand upright on its six legs, after being brought back to

room temperature following a cold-induced coma. Sorting and sexing of flies prior to the experiments was conducted using CO_2-anesthetization. Flies were always allowed to recover in food vials for at least 24h before the onset of the cold shock. Age of flies was two to six days at the onset of the cold shock. For the cold shock, they were flipped into empty plastic vials, immediately put into an ice-water bath and kept in the dark for the entire duration (7h) of the cold shock. Experiments were conducted with ~10 flies per experimental vial. Recovery status was monitored for 90 minutes in 5 minute intervals at ambient temperatures (22±1 °C). Living flies that had not recovered after 90 minutes were assigned a recovery time of 95 minutes. Dead flies were excluded from the analysis. Mortality during the experiments, however, was very low (below 1%).

5.3 Plasticity experiments

CCRT experiments were conducted at four distinct experimental conditions:

1. Standard (as described above for the initial phenotyping)

2. Acclimation (rearing like standard with an additional acclimation treatment of 12h at 6°C prior to the cold shock)

3. Cold rearing (fly culture in an incubator at 17°C)

4. Cold rearing + acclimation (fly culture at 17°C plus an additional acclimation treatment of 12h at 6°C prior to the cold shock)

To account for slower growth rates at 17°C those flies were allowed to recover for two days after CO_2-anesthetization and sorting instead of one day for flies reared at 22°C.

5.4 Repeated cold shocks

To assess the ability to fully recover from a cold shock, flies were subjected to two or three cold shocks of 7h in a row. After the initial calling of CCRT following the standard procedure described above flies were flipped back to their prior food vials and kept at 22°C before they were subjected to the next 7h cold shock, which took place 24h after the onset of the first cold shock.

5.5 Assessment of general fitness parameters

All experiments were conducted at standard conditions (21°C ± 1°C, 25-50% air humidity) in parallel for five European and five African strains in eightfold replication, though few replicates had to be excluded for individual strains due to various reasons (accidental loss of vials during flipping, reduced growth so that not enough flies were available, etc). The number of tested replicates per strain is indicated in the results figure.

Egg laying capacity: Fifteen female and 15 male flies of five days of age were sorted using CO_2-anesthetization and put into a fine wire mesh mating cage (ø = 8.1cm; height = 9.9cm) on a nutrient medium plate containing sugar beet molasses, agar-agar for medium consistency, propionic acid and nipagin as preservatives, and an additional 50µl of 20% live yeast solution applied on the surface ~30 minutes before the start of the experiment to allow drying. The plate was sealed to the cage with parafilm. After three days the plate was replaced with a fresh nutrient plate without yeast to alleviate the subsequent counting of eggs. After another 24h the flies were removed and the number of laid eggs was counted under a microscope.

Egg hatchability: The number of hatched larvae was counted three days after the removal of parental flies and checked again on day five.

Larva-to-adult viability: Three days after oviposition 50 larvae were manually transferred with a needle to a 50ml vial containing standard fly medium. The number of eclosed flies was counted 18 days later.

CCRT: After counting, ~10 female and ~10 male flies were subjected to a 7h cold shock. On day 19, CCRT was assessed following the standard procedure described above.

Longevity: Remaining male flies and male flies from after the CCRT experiment were transferred to separate fresh 50ml standard vials. Survival was monitored in two day intervals until all flies were dead. Flies were not transferred to new vials during the course of this experiment, putatively introducing desiccation as a major influencing factor.

5.6 Evaluation of phenotypic data

Statistical analysis was performed with Microsoft Excel 2007 and R (version 3.2.1) [R Core Team, 2015]. Unless noted otherwise, CCRT was averaged over all experiments of the respective conditions and both sexes. For the multifactorial ANOVA CCRT-values were log-transformed to obtain a normal distribution. Normality was confirmed by visual inspection of a Q-Q plot.

5.7 Sample preparation, RNA extraction and RNA sequencing

Extractions were performed in January and February of 2014. Samples were obtained at four distinct conditions: Without cold treatment at room

temperature, 3.5h after beginning of the cold shock, and 15 and 90 minutes after flies have been brought back to room temperature following a 7h cold shock at 0°C in an ice-water bath. For every strain-condition-combination two biological replicates were produced roughly two weeks apart and with flies stemming from different vials to account for vial effects. For every sample 16 male flies at five days of age were frozen in liquid nitrogen. Nucleic acid extraction was performed using Epicentre MasterPure Complete DNA and RNA Purification Kit according to the manufacturer's protocol without DNAse treatment. Sample quality was assessed using Nanodrop (Thermo Fisher Scientific) and Bioanalyzer (Agilent Technologies). All samples were free from considerable amounts of contaminants and showed no signs of RNA degradation. Samples were stored at minus 80°C prior to shipment to a sequencing company (GATC Biotech, Konstanz, Germany) for PolyA-enrichment, random primed cDNA synthesis, library preparation and sequencing on an Illumina HiSeq2000 yielding >>20 million 51bp single reads per sample with an average Phred score above 30 for the bps with lowest quality.

5.8 Read mapping

The obtained reads were mapped to the *D. melanogaster* transcriptome (FlyBase release 5.57 [St Pierre et al., 2014]) using NextGenMap [Sedlazeck et al., 2013]. For every read, only the first best hit was counted. All reads mapping to multiple transcripts of a single gene were collapsed. The per sample library size ranges from 16 to 37 million reads. Average library size is

25.4 million reads. All reads that did not map to any transcript were then mapped to other features of the *D. melanogaster* genome. The reads that did not map to any of these as well were considered unmapped.

5.9 Ambiguous mapping

With special regard to *Hsp70*, which is the gene with the highest cold-induced fold-change in expression, it must be noted that there are actually six different copies of *Hsp70* annotated in FlyBase 5.57. It would, however, require sophisticated approaches to be able to tell them apart due to their extremely high sequence similarity. Thus, they are commonly treated as a single gene in studies of gene expression [Sinclair et al., 2007; Colinet et al., 2010c]. The issue of ambiguous mapping further extends to other gene families and virtually to any two genes that share stretches of identical transcript sequence. Since within-gene-family-differences in expression are not of prime concern in this study and since this should affect both populations in a similar fashion, it is assumedly of little consequence for the overall results.

5.10 Calling of differentially expressed genes and enriched gene properties

Differentially expressed genes (DEG) were called using the DESeq2 package (version 1.6.3) [Love et al., 2014] for R (version 3.2.1) [R Core Team, 2015] and a 5% FDR cutoff based on Benjamini-Hochberg adjusted P-values [Benjamini & Hochberg, 1995]. A model with three factors was used, namely continent (Europe, Africa), condition (RT, CS, rec15, rec90), and an

interaction term between the two. Thus, there are eight samples per continent-condition-combination, consisting of 4 strains in twofold biological replication. For the multilevel contrasts of the factor condition (RT vs. CS, RT vs. rec15, RT vs. rec90) the Benjamini-Hochberg-correction was performed over all P-values of the three tests combined. The number of significant genes was maximized by applying independent filtering [Bourgon et al., 2010] with the mean expression over all 64 samples as independent filter criterion. The same correction approach was also applied to the multilevel interaction contrasts.
GO term enrichment was calculated using GOrilla [Eden et al., 2009] against the background of all annotated genes in the genome and with regard to multiple testing (<5% FDR) [Benjamini & Hochberg, 1995].

5.11 qRT-PCR

Each sample was prepared with approximately eight male flies at five days of age. Four biological replicates were produced for every strain-timepoint-combination. RNA extraction was performed as described above, with the exception of an additional 1h DNAse I treatment at 37°C to get rid of genomic DNA. Absence of genomic DNA was determined using a highly sensitive Phusion polymerase (NEB) and a set of primers that only amplifies genomic DNA (X-01435+: 5'-TGC GAA ACA GGT ACA AGT-3'; X-01435-: 5'-GGA TTC GTG AAC GGG AAA-3'). Furthermore, for a few samples noRT controls were run in the qPCR. cDNA was synthesized using random hexamers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Gene specific primer sequences for qPCR were

created with quantprime [Arvidsson et al., 2008]. All primers were obtained from Metabion (Planegg, Germany). The ribosomal genes *RpL32* and *RpS20* were used as reference genes [Voigt et al., 2015]. Primer sequences and annealing temperatures are shown in Table 5. SYBR green Master Mix (Bio-Rad) was used as reaction and detection reagent on a Real-Time thermal cycler CFX96 (Bio-Rad). Every sample was assessed in threefold technical replication. Gene expression was quantified using the ΔΔCt method [Hellemans et al., 2007].

Table 5 | Primers for qPCR

Gene	Forward primer	Reverse primer	Annealing temperature
RpL32	ATCGTGAAGAAGCGCACCAAGC	TTGCGCCATTTGTGCGACAG	62.1 °C
RpS20	TTCGCATCACCACCCGTAAGAC	TTGTGGATTCTCATCTGGAAGCG	61.3 °C
Frost	TGCAGGAACAGAGGTGGAATAGC	TGACCCTGACCGTTGCCATTTG	59.5 °C
Hsp23	AGCGAACTGGTGGTCAAAGTGC	ATCTTCGCGCTCCTCATGGTTG	63.0 °C
CG10912	TCCTGCTGGACTGCGATAAACAG	GCTTGGAATATGTCGGACCCTCAG	61.0 °C
brinker	TGCGAGGACATCATCCGTCAAC	TCAGGTTTGTGGGCGCAGTATC	62.2 °C
smp-30	ACCGTCTTCAAGGTCAATCCAAGC	AAAGCCACCGAGGTGATTTGGG	60.9 °C
CG12164	GCATGAGATCCCTAAGTCTGTTGC	TCTTCCACACCTGGACCCAATC	65.0 °C
TotA	TTCCGGTTTGCTTCAGCGTTCC	AGCAGCAGTGCAAAGCACATAAG	58.8 °C
TotC	TCTACTATGCCTTGCCCTGCTC	ATCTTCGATTCGGCGTCGTTGG	62.4 °C

5.12 Functional assessment of the knockdown of individual genes

Fly lines containing gene-specific inverted repeats (IR) were ordered from the VDRC [Dietzl et al., 2007] and belong either to the GD or KK library. In these lines the IR is under the control of a GAL4-inducible promoter (=upstream activating sequence (UAS)), i.e. only expressed in presence of GAL4. To activate expression of the IR and, thus, the RNAi-mediated knockdown of the respective gene, young males of the IR lines and of their respective controls

(w^{1118} for the GD library and KK60100 for the KK library) were crossed to young virgin females of two driver lines that ubiquitously express GAL4 under the control of either the Act5C- (Act5C-GAL4 / CyO) or the α-Tubulin-promoter (UAS-Dicer2; αTub-GAL4 / TM3, Kr-GFP). Progeny was screened for straight vs. curly wings (Act5C) or normal vs. stubble bristles (αTub) to obtain the desired cross. The efficiency of the knockdown was determined via qPCR. The knockdown- and control-crosses were phenotyped in parallel for changes in cold tolerance. CCRT was determined as described above. For the survival assays, flies were transferred back to food vials after the recovery experiments and kept at 22°C±1°C in separate vials for each sex. The proportion of living flies was counted roughly 24h after the end of the cold shock. Mortality clearly peaks within this period of time, as subsequent checks at 48h revealed identical numbers of fatalities in the great majority of cases.

Appendix

Table A1 | CCRT (12h CS) using the αTub driver and the KK-library

gene/sex	avg. CCRT	SD	n	p-Value*	p-Value corrected**
control♀	39.4	4.0	15		
control♂	41.5	4.0	15		
Frost♀	42.1	6.0	17	0.1395	1.0000
Frost♂	39.2	4.4	17	0.1427	1.0000
CG12164♀	40.5	4.3	3	0.6605	1.0000
CG12164♂	42.4	5.5	4	0.6996	1.0000
Hsp23♀	39.8	6.1	4	0.8707	1.0000
Hsp23♂	38.1	3.5	4	0.1420	1.0000

n = number of replicate experiments with ~10 flies each
*two-sided T-test for difference to respective control
**Bonferroni correction to account for multiple testing (73 comparisons in total)

Table A2 | Survival (12h CS) using the αTub driver and the KK-library

gene/sex	avg. 24h survival	SD	n	p-Value*	p-Value corrected**
control♀	87.3%	12.8%	15		
control♂	93.3%	12.6%	15		
Frost♀	86.4%	12.6%	17	0.8394	1.0000
Frost♂	98.9%	3.0%	17	0.0857	1.0000
CG12164♀	78.8%	18.9%	3	0.3416	1.0000
CG12164♂	88.1%	8.6%	4	0.4516	1.0000
Hsp23♀	79.5%	15.5%	4	0.3161	1.0000
Hsp23♂	97.7%	4.5%	4	0.5099	1.0000

n = number of replicate experiments with ~10 flies each
*two-sided T-test for difference to respective control
**Bonferroni correction to account for multiple testing (41 comparisons in total)

Table A3 | CCRT (9h CS) using the αTub driver and the KK-library

gene/sex	avg. CCRT	SD	n	p-Value*	p-Value corrected**
control♀	37.3	6.1	43		
control♂	38.3	6.2	43		
CG17752♀	35.2	6.4	18	0.2187	1.0000
CG17752♂	34.6	4.5	18	**0.0260**	1.0000
Hsp23♀	35.3	4.3	12	0.2786	1.0000
Hsp23♂	34.1	3.5	12	**0.0301**	1.0000
brinker♀	37.9	6.2	14	0.7457	1.0000
brinker♂	40.9	7.2	14	0.2027	1.0000
Frost♀	37.5	10.0	18	0.9330	1.0000
Frost♂	35.9	5.5	18	0.1570	1.0000
fok♀	39.8	4.8	23	0.0910	1.0000
fok♂	39.2	5.4	23	0.5564	1.0000
pirk♀	38.4	4.6	21	0.4611	1.0000
pirk♂	38.8	4.8	21	0.7507	1.0000
CG31689♀	41.0	7.3	12	0.0821	1.0000
CG31689♂	38.5	11.0	11	0.9289	1.0000
CG5953♀	38.9	14.2	27	0.5124	1.0000
CG5953♂	39.1	9.3	27	0.6895	1.0000
CG12164♀	43.9	3.5	2	0.1424	1.0000
CG12164♂	39.8	3.5	2	0.7452	1.0000
CG13510♀	37.0	8.4	17	0.8876	1.0000
CG13510♂	37.3	5.8	17	0.5792	1.0000
Lnk♀	36.8	6.0	25	0.7247	1.0000
Lnk♂	35.7	4.4	25	0.0669	1.0000
Hsp26♀	37.5	8.3	18	0.9257	1.0000
Hsp26♂	33.6	3.5	18	**0.0035**	0.2545
Hsp68♀	37.6	3.2	6	0.9216	1.0000

Hsp68 knockdown is lethal for males

n = number of replicate experiments with ~10 flies each

*two-sided T-test for difference to respective control

**Bonferroni correction to account for multiple testing (73 comparisons in total)

Table A4 | Survival (9h CS) using the αTub driver and the KK-library

gene/sex	avg. 24h survival	SD	n	p-Value*	p-Value corrected**
control♀	69.5%	29.0%	39		
control♂	78.8%	28.6%	43		
CG17752♀	66.5%	27.2%	18	0.7089	1.0000
CG17752♂	94.2%	9.6%	18	**0.0306**	1.0000
Hsp23♀	75.2%	22.3%	11	0.5507	1.0000
Hsp23♂	97.7%	5.7%	12	**0.0277**	1.0000
brinker♀	76.4%	19.9%	14	0.4184	1.0000
brinker♂	61.7%	35.6%	14	0.0738	1.0000
Frost♀	68.6%	35.5%	15	0.9236	1.0000
Frost♂	93.3%	14.1%	18	**0.0453**	1.0000
fok♀	67.6%	26.7%	22	0.8011	1.0000
fok♂	94.8%	8.3%	23	**0.0112**	0.4590
pirk♀	71.4%	25.9%	21	0.8011	1.0000
pirk♂	91.0%	19.3%	21	0.0815	1.0000
CG31689♀	68.6%	23.8%	12	0.9195	1.0000
CG31689♂	85.9%	18.9%	11	0.4369	1.0000
CG5953♀	61.8%	34.6%	24	0.3464	1.0000
CG5953♂	87.4%	21.6%	27	0.1843	1.0000
CG12164♀	45.5%	25.7%	2	0.2587	1.0000
CG12164♂	81.8%	12.9%	2	0.8834	1.0000
CG13510♀	77.6%	29.1%	13	0.3875	1.0000
CG13510♂	92.3%	11.3%	17	0.0643	1.0000
Lnk♀	84.4%	14.8%	25	**0.0211**	0.8645
Lnk♂	96.7%	6.9%	25	**0.0031**	0.1291
Hsp26♀	52.5%	20.8%	18	**0.0293**	1.0000
Hsp26♂	91.2%	12.3%	18	0.0836	1.0000
Hsp68♀	85.9%	10.9%	6	0.1825	1.0000

Hsp68 knockdown is lethal for males

n = number of replicate experiments with ~10 flies each

*two-sided T-test for difference to respective control

**Bonferroni correction to account for multiple testing (41 comparisons in total)

Table A5 | CCRT (9h CS) using the αTub driver and the GD-library

gene/sex	avg. CCRT	SD	n	p-Value*	p-Value corrected**
control♀	36.1	11.3	13		
control♂	38.1	5.0	13		
Hsp22♀	33.1	4.9	28	0.2348	1.0000
Hsp22♂	36.3	5.6	28	0.3162	1.0000
Hsp70♀	33.7	7.4	26	0.4233	1.0000
Hsp70♂	35.2	4.5	26	0.0740	1.0000
Diedel3♀	31.3	6.3	26	0.0952	1.0000
Diedel3♂	35.9	6.1	27	0.2533	1.0000
CG7130♀	28.9	4.3	27	**0.0052**	0.3790
CG7130♂	31.3	4.1	27	**0.0001**	**0.0037**
CG14205♀	29.9	5.5	26	**0.0243**	1.0000
CG14205♂	33.4	4.9	26	**0.0083**	0.6072

n = number of replicate experiments with ~10 flies each
*two-sided T-test for difference to respective control
**Bonferroni correction to account for multiple testing (73 comparisons in total)

Table A6 | survival (9h CS) using the αTub driver and the GD-library

gene/sex	avg. 24h survival	SD	n	p-Value*	p-Value corrected**
control♀	83.8%	33.0%	9		
control♂	90.9%	27.8%	13		
Hsp22♀	82.2%	24.8%	24	0.8781	1.0000
Hsp22♂	97.6%	6.4%	28	0.2315	1.0000
Hsp70♀	75.1%	29.7%	22	0.4774	1.0000
Hsp70♂	94.2%	10.8%	26	0.5928	1.0000
Diedel3♀	87.0%	22.4%	23	0.7595	1.0000
Diedel3♂	87.9%	19.3%	27	0.6963	1.0000
CG7130♀	93.6%	17.2%	23	0.2778	1.0000
CG7130♂	99.0%	3.9%	27	0.1412	1.0000
CG14205♀	86.0%	22.1%	22	0.8299	1.0000
CG14205♂	98.8%	4.3%	26	0.1579	1.0000

n = number of replicate experiments with ~10 flies each
*two-sided T-test for difference to respective control
**Bonferroni correction to account for multiple testing (41 comparisons in total)

Table A7 | CCRT (7h CS) using the Act5C driver and the KK-library

gene/sex	avg. CCRT	SD	n	p-Value*	p-Value corrected**
control♀	30.4	7.5	9		
control♂	35.6	4.4	9		
Frost♀	30.5	6.6	9	0.9658	1.0000
Frost♂	33.7	6.2	9	0.4690	1.0000
CG12164♀	31.4	7.3	9	0.7800	1.0000
CG12164♂	30.8	5.5	9	0.0579	1.0000
TotA♀	33.3	7.2	9	0.4107	1.0000
TotA♂	29.4	4.2	9	**0.0077**	0.5649
Hsp23♀	32.0	7.6	7	0.6775	1.0000
Hsp23♂	34.4	2.4	5	0.6029	1.0000

n = number of replicate experiments with ~10 flies each
*two-sided T-test for difference to respective control
**Bonferroni correction to account for multiple testing (73 comparisons in total)

Table A8 | CCRT (15h CS) using the Act5C driver and the KK-library

gene/sex	avg. CCRT	SD	n	p-Value*	p-Value corrected**
control♀	61.5	6.9	14		
control♂	66.8	5.9	14		
CG8778♀	57.7	6.5	14	0.1561	1.0000
CG8778♂	64.8	5.9	14	0.3779	1.0000
CG13510♀	63.6	8.6	14	0.4641	1.0000
CG13510♂	70.3	5.0	14	0.1027	1.0000
control♀	58.5	7.1	12		
control♂	66.6	6.4	12		
brv3♀	53.7	6.1	12	0.1010	1.0000
brv3♂	64.6	9.0	12	0.5575	1.0000
irk3♀	61.8	7.1	12	0.5296	1.0000
irk3♂	67.4	4.5	12	0.7259	1.0000
KCNQ♀	57.4	4.5	12	0.6518	1.0000
KCNQ♂	67.9	2.9	12	0.5296	1.0000
control♀	58.1	5.0	10		
control♂	62.7	6.5	10		
Trap1♀	54.6	3.7	10	0.0922	1.0000
Trap1♂	62.2	6.1	10	0.8617	1.0000
sas♀	55.2	6.0	10	0.2475	1.0000
sas♂	64.6	6.0	10	0.5237	1.0000

Three different controls correspond to three different experimenters
n = number of replicate experiments with ~10 flies each
*two-sided T-test for difference to respective control
**Bonferroni correction to account for multiple testing (73 comparisons in total)

Table A9 | CCRT (18h CS) using the Act5C driver and the KK-library

gene/sex	avg. CCRT	SD	n	p-Value*	p-Value corrected**
control♀	65.5	9.2	21		
control♂	83.9	10.5	21		
Frost♀	71.3	11.4	23	0.0712	1.0000
Frost♂	095.2	11.3	23	**0.0014**	0.1008
CG12164♀	079.7	13.2	10	**0.0016**	0.1185
CG12164♂	103.8	07.5	10	**0.0000**	**0.0007**
brv3♀	070.4	12.7	7	0.2769	1.0000
brv3♂	082.8	06.7	7	0.8140	1.0000
TotA♀	081.0	11.7	14	**0.0001**	**0.0085**
TotA♂	090.1	14.2	14	0.1421	1.0000
Hsp23♀	064.7	09.5	20	0.7815	1.0000
Hsp23♂	083.1	10.1	20	0.8132	1.0000

n = number of replicate experiments with ~10 flies each
*two-sided T-test for difference to respective control
**Bonferroni correction to account for multiple testing (73 comparisons in total)

Table A10 | Survival (18h CS) using the Act5C driver and the KK-library

gene/sex	avg. 24h survival	SD	n	p-Value*	p-Value corrected**
control♀	057.3%	42.1%	17		
control♂	094.0%	07.9%	17		
Frost♀	072.1%	29.3%	20	0.2169	1.0000
Frost♂	098.6%	04.4%	20	**0.0301**	1.0000
CG12164♀	063.9%	31.1%	10	0.6715	1.0000
CG12164♂	100.0%	00.0%	10	**0.0244**	1.0000
brv3♀	070.1%	29.6%	7	0.4733	1.0000
brv3♂	080.1%	17.6%	7	**0.0132**	0.5414
TotA♀	76.6%	21.5%	14	0.1322	1.0000
TotA♂	91.4%	17.4%	14	0.5947	1.0000
Hsp23♀	61.0%	33.9%	17	0.7824	1.0000
Hsp23♂	97.8%	4.9%	16	0.1050	1.0000

n = number of replicate experiments with ~10 flies each
*two-sided T-test for difference to respective control
**Bonferroni correction to account for multiple testing (41 comparisons in total)

Bibliography

1. Andersen JL, Manenti T, Sorensen JG, MacMillan HA, Loeschke V, Overgaard J. How to assess *Drosophila* cold tolerance: chill coma temperature and lower lethal temperature are the best predictors of cold distribution limits. 2015;29:55-65.
2. Arvidsson S, Kwasniewski M, Riaño-Pachón DM, Mueller-Roeber B. QuantPrime--a flexible tool for reliable high-throughput primer design for quantitative PCR. BMC Bioinformatics. 2008;9:465.
3. Ayrinhac A, Debat V, Gibert P, Kister AG, Legout H, Moreteau B, Vergilino R, David JR. Cold adaptation in geographical populations of *Drosophila melanogaster* : phenotypic plasticity is more important than genetic variability. Funct Ecol. 2004;18:700-6.
4. Begun DJ, Aquadro CF. African and North American populations of *Drosophila melanogaster* are very different at the DNA level. 1993;365:548-50.
5. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Royal Stat Soc B. 1995;57:289-300.
6. Bourgon R, Gentleman R, Huber W. Independent filtering increases detection power for high-throughput experiments. Proc Natl Acad Sci U S A. 2010;107:9546-51.
7. Bubliy OA, Loeschcke V. Effect of low stressful temperature on genetic variation of five quantitative traits in *Drosophila melanogaster*. Heredity (Edinb). 2002;89:70-5.
8. Caracristi G, Schlötterer C. Genetic differentiation between American and European *Drosophila melanogaster* populations could be attributed to admixture of African alleles. Mol Biol Evol. 2003;20:792-9.
9. Chanderbali AS, Yoo MJ, Zahn LM, Brockington SF, Wall PK, Gitzendanner MA, Albert VA, Leebens-Mack J, Altman NS, Ma H, dePamphilis CW, Soltis DE, Soltis PS. Conservation and canalization of

gene expression during angiosperm diversification accompany the origin and evolution of the flower. Proc Natl Acad Sci U S A. 2010;107:22570-5.

10. Chen J, Nolte V, Schlötterer C. Temperature-Related Reaction Norms of Gene Expression: Regulatory Architecture and Functional Implications. Mol Biol Evol. 2015;32:2393-402.
11. Clarke A. The influence of climate change on the distribution and evolution of organisms. In: Johnston IA, Bennett AF, editors: Animals and Temperature: Phenotypic and Evolutionary Adaptation. Cambridge: Cambridge University Press; 1996. p. 377-407.
12. Colinet H, Lee SF, Hoffmann A. Knocking down expression of *Hsp22* and *Hsp23* by RNA interference affects recovery from chill coma in *Drosophila melanogaster*. J Exp Biol. 2010;213:4146-50. (≙ 2010b)
13. Colinet H, Lee SF, Hoffmann A. Functional characterization of the *Frost* gene in *Drosophila melanogaster*: importance for recovery from chill coma. PLoS One. 2010;5:e10925. (≙ 2010a)
14. Colinet H, Lee SF, Hoffmann A. Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult *Drosophila melanogaster*. FEBS Journal. 2010;277:174-85. (≙ 2010c)
15. Colinet H, Nguyen TT, Cloutier C, Michaud D, Hance T. Proteomic profiling of a parasitic wasp exposed to constant and fluctuating cold exposure. Insect Biochem Mol Biol. 2007;37:1177-88.
16. Costa M, Calleja M, Alonso CR, Simpson P. The bristle patterning genes *hairy* and *extramacrochaetae* regulate the development of structures required for flight in Diptera. Dev Biol. 2014; 388:205-15.
17. Daborn PJ, Lumb C, Boey A, Wong W, Ffrench-Constant RH, Batterham P. Evaluating the insecticide resistance potential of eight *Drosophila melanogaster* cytochrome P450 genes by transgenic over-expression. Insect Biochem Mol Biol. 2007;37:512-9.
18. Daborn PJ, Yen JL, Bogwitz MR, Le Goff G, Feil E, Jeffers S, Tijet N, Perry T, Heckel D, Batterham P, Feyereisen R, Wilson TG, Ffrench-Constant RH. A single p450 allele associated with insecticide resistance in *Drosophila*. Science. 2002;297:2253-6.

19. David JR, Capy P. Genetic variation of *Drosophila melanogaster* natural populations. Trends Genet. 1988;4:106-11.
20. David JR, Gibert P, Pla E, Petavy G, Karan D, Moreteau B. Cold stress tolerance in *Drosophila*: analysis of chill coma recovery in *D. Melanogaster*. J Therm Biol. 1998;23:291-9.
21. Dennis AB, Dunning LT, Sinclair BJ, Buckley TR. Parallel molecular routes to cold adaptation in eight genera of New Zealand stick insects. Sci Rep. 2015;5:13965.
22. Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, Couto A, Marra V, Keleman K, Dickson BJ. A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Nature. 2007;448:151-6.
23. Duchen P, Zivkovic D, Hutter S, Stephan W, Laurent S. Demographic inference reveals African and European admixture in the North American *Drosophila melanogaster* population. Genetics. 2013;139:291-301.
24. Duggan DJ, Bittner M, Chen Y, Meltzer P, Trent JM. Expression profiling using cDNA microarrays. Nature genetics. 1999;21:10-4.
25. Eden E, Navon R, Steinfeld I, Lipson D, Yakhini Z. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. BMC Bioinformatics. 2009;10:48.
26. Ellegren H, Parsch J. The evolution of sex-biased genes and sex-biased gene expression. Nat Rev Genet. 2007;8:689-98.
27. Endler JA. Geographic variation, speciation, and clines. Monogr Popul Biol. 1977;10:1-246.
28. Fan CY, Lee S, Cyr DM. Mechanisms for regulation of *Hsp70* function by *Hsp40*. Cell Stress Chaperones. 2003;8:309-16.
29. Flatt T. The evolutionary genetics of canalization. Q Rev Biol. 2005;80:287-316.
30. Forsman A. Rethinking phenotypic plasticity and its consequences for individuals, populations and species. Heredity. 2015;115:276-84.
31. Garland TGJ. Trade-offs. Current Biology. 2014;2:R60-1

32. Ghalambor CK, Hoke KL, Ruell EW, Fischer EK, Reznick DN, Hughes KA. Non-adaptive plasticity potentiates rapid adaptive evolution of gene expression in nature. Nature. 2015;525:372-5.
33. Gibert P, Moreteau B, Petavy G, Karan D, David JR. Chill-coma tolerance, a major climatic adaptation among *Drosophila* species. Evolution. 2001;55:1063-8.
34. Glinka S, Ometto L, Mousset S, Stephan W, De Lorenzo D. Demography and natural selection have shaped genetic variation in *Drosophila melanogaster*: a multi-locus approach. Genetics. 2003;165:1269-78.
35. Goto SG. A novel gene that is up-regulated during recovery from cold shock in *Drosophila melanogaster*. Gene. 2001;270:259-64.
36. Goto SG. Expression of *Drosophila* homologue of senescence marker protein-30 during cold acclimation. J Insect Physiol. 2000;46:1111-20.
37. Graveley BR, Brooks AN, Carlson JW, Duff MO, Landolin JM, Yang L, Artieri CG, van Baren MJ, Boley N, Booth BW, Brown JB, Cherbas L, Davis CA, Dobin A, Li R, Lin W, Malone JH, Mattiuzzo NR, Miller D, Sturgill D, Tuch BB, Zaleski C, Zhang D, Blanchette M, Dudoit S, Eads B, Green RE, Hammonds A, Jiang L, Kapranov P, Langton L, Perrimon N, Sandler JE, Wan KH, Willingham A, Zhang Y, Zou Y, Andrews J, Bickel PJ, Brenner SE, Brent MR, Cherbas P, Gingeras TR, Hoskins RA, Kaufman TC, Oliver B, Celniker SE. The developmental transcriptome of *Drosophila melanogaster*. Nature. 2011;47:473-79.
38. Hatle JD, Borst DW, Juliano SA. Plasticity and canalization in the control of reproduction in the lubber grasshopper. Integr Comp Biol. 2003;43:653-45.
39. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 2007;8:R19.
40. Hoffmann JA, Hetru C, Reichhart JM. The humoral antibacterial response of *Drosophila*. FEBS Lett. 1993;325:63-6.

41. Hoffmann AA, Weeks AR. 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. 2007;129:133-47.
42. Huang W, Richards S, Carbone MA, Zhu D, Anholt RR, Ayroles JF, Duncan L, Jordan KW, Lawrence F, Magwire MM, Warner CB, Blankenburg K, Han Y, Javaid M, Jayaseelan J, Jhangiani SN, Muzny D, Ongeri F, Perales L, Wu YQ, Zhang Y, Zou X, Stone EA, Gibbs RA, Mackay TF. Epistasis dominates the genetic architecture of Drosophila quantitative traits. Proc Natl Acad Sci U S A. 2012;109:15553-9.
43. Hutter S, Saminadin-Peter SS, Stephan W, Parsch J. Gene expression variation in African and European populations of *Drosophila melanogaster*. Genome Biol. 2008;9:R12.
44. Kayukawa T, Ishikawa Y. Chaperonin contributes to cold hardiness of the onion maggot *Delia antiqua* through repression of depolymerization of actin at low temperatures. PLoS One. 2009;4:e8277.
45. Keller A. *Drosophila melanogaster's* history a a human commensal. Curr Biol. 2007;17:R77-81.
46. Kelty JD, Lee RE Jr. Induction of rapid cold hardening by cooling at ecologically relevant rates in *Drosophila melanogaster*. J Insect Physiol. 1999;45:719-26.
47. Kim E, Choi Y, Lee S, Seo Y, Yoon J, Baek K. Characterization of the *Drosophila melanogaster retinin* gene encoding a cornea-specific protein. Insect Mol Biol. 2008;17:537-43.
48. Kim LK, Choi UY, Cho HS, Lee JS, Lee WB, Kim J, Jeong K, Shim J, Kim-Ha J, Kim YJ. Down-regulation of NF-kappa B target genes by the AP-1 and STAT complex during the innate immune response in *Drosophila*. PLoS Biol. 2007;5:e238.
49. Kim M, Robich RM, Rinehart JP, Denlinger DL. Upregulation of two actin genes and redistribution of actin during diapause and cold stress in the northern house mosquito, *Culex pipiens*. J Insect Physiol. 2006;52:1226-33.

50. King MC, Wilson AC. Evolution at two levels in humans and chimpanzees. Science. 1975;188:107-16.
51. Kriegenburg F, Ellgaard L, Hartmann-Petersen R. Molecular chaperones in targeting misfolded proteins for ubiquitin-dependent degradation. FEBS J. 2012;279:532-42.
52. Lachaise D, Silvain JF. How two Afrotropical endemics made two cosmopolitan human commensals: the *Drosophila melanogaster–D. simulans* palaeogeographic riddle. Genetica. 2004;120:17-39.
53. Levine MT, Eckert ML, Begun DJ. Whole-genome expression plasticity across tropical and temperate *Drosophila melanogaster* populations from Eastern Australia. Mol Biol Evol. 2011;28:249-56.
54. Lewin B. Genes VII. Oxford University Press. 2000.
55. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011;12:323.
56. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15:550.
57. Ma S, Huang Y, van Huystee RB. Improved plant RNA stability in storage. Anal Biochem. 2004;326:122-4.
58. Mackay TF, Richards S, Stone EA, Barbadilla A, Ayroles JF, Zhu D, Casillas S, Han Y, Magwire MM, Cridland JM, Richardson MF, Anholt RR, Barrón M, Bess C, Blankenburg KP, Carbone MA, Castellano D, Chaboub L, Duncan L, Harris Z, Javaid M, Jayaseelan JC, Jhangiani SN, Jordan KW, Lara F, Lawrence F, Lee SL, Librado P, Linheiro RS, Lyman RF, Mackey AJ, Munidasa M, Muzny DM, Nazareth L, Newsham I, Perales L, Pu LL, Qu C, Ràmia M, Reid JG, Rollmann SM, Rozas J, Saada N, Turlapati L, Worley KC, Wu YQ, Yamamoto A, Zhu Y, Bergman CM, Thornton KR, Mittelman D, Gibbs RA. The *Drosophila melanogaster* Genetic Refernce Panel. Nature. 2012;482:173-8.
59. MacMillan HA, Sinclair BJ. Mechanisms underlying insect chill-coma. J Insect Physiol. 2011;57:12-20.

60. MacMillan HA, Williams CM, Staples JF, Sinclair BJ. Reestablishment of ion homeostasis during chill-coma recovery in the cricket *Gryllus pennsylvanicus*. Proc Natl Acad Sci U S A. 2012;109:20750-5.
61. MacMillan HA, Ferguson LV, Nicolai A, Donini A, Staples JF, Sinclair BJ. Parallel ionoregulatory adjustements underlie phenotypic plasticity and evolution of *Drosophila* cold tolerance. J Exp Biol. 2015;218:423-32.
62. Manu, Surkova S, Spirov AV, Gursky VV, Janssens H, Kim AR, Radulescu O, Vanario-Alonso CE, Sharp DH, Samsonova M, Reinitz J. Canalization of gene expression in the *Drosophila* blastoderm by gap gene cross regulation. PLoS Biol. 2009;7:e1000049.
63. Marshall KE, Sinclair BJ. The impacts of repeated cold exposure on insects. J Exp Biol. 2012;215:1607-13.
64. Müller L, Hutter S, Stamboliyska R, Saminadin-Peter SS, Stephan W, Parsch J. Population transcriptomics of *Drosophila melanogaster* females. BMC Genomics. 2011;12:81.
65. Nielsen MM, Overgaard J, Sorensen JG, Holmstrup M, Justensen J, Loeschke V. Role of HSF activation for resistance to heat, cold and high-temperature knock-down. J Insect Physiol. 2005;51:1320-9.
66. Orvar BL, Sangwan V, Omann F, Dhindsa RS. Early steps in cold sensing by plant cells: the role of actin cytoskeleton and membrane fluidity. Plant J. 2000;23:785-94.
67. Paparazzo F, Tellier A, Stephan W, Hutter S. Survival Rate and Transcriptional Response upon Infection with the Generalist Parasite *Beauveria bassiana* in a World-Wide Sample of *Drosophila melanogaster*. PLoS One. 2015;10:e0132129.
68. Pearson K. On Lines and Planes of Closest Fit to Systems of Points in Space. Philos Mag. 1901;2:559-572.
69. Pool JE, Corbett-Detig RB, Sugino RP, Stevens KA, Cardeno CM, Crepeau MW, Duchen P, Emerson JJ, Saelao P, Begun DJ, Langley CH. Population Genomics of sub-saharan *Drosophila melanogaster*: African diversity and non-African admixture. PLoS Genet. 2012;8:e1003080.

70. Pool JE, Braun DT, Lack JB. Parallel evolution of cold tolerance within *Drosophila melanogaster*. Mol Biol Evol. 2016. Epub ahead of print.
71. Qin W, Neal SJ, Robertson RM, Westwood JT, Walker VK. Cold hardening and transcriptional change in *Drosophila melanogaster*. Insect Mol Biol. 2005;14:607-13.
72. R Core Team. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2015. URL https://www.R-project.org/.
73. Saunders DS, Henrich VC, Gilbert LI. Induction of diapause in *Drosophila melanogaster*: photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. Proc Natl Acad Sci U S A. 1989;86:3748-52.
74. Schou MF, Kristensen TN, Kellermann V, Schlötterer C, Loeschcke V. A *Drosophila* laboratory evolution experiment points to low evolutionary potential under increased temperatures likely to be experienced in the future. J Evol Biol. 2014;27:1859-68.
75. Sedlazeck FJ, Rescheneder P, von Haeseler A. NextGenMap: fast and accurate read mapping in highly polymorphic genomes. Bioinformatics. 2013;29:2790-1.
76. Shaw JR, Hampton TH, King BL, Whitehead A, Galvez F, Gross RH, Keith N, Notch E, Jung D, Glaholt SP, Chen CY, Colbourne JK, Stanton BA. Natural selection canalizes expression variation of environmentally induced plasticity-enabling genes. Mol Biol Evol. 2014;31:3002-15.
77. Siegal ML, Bergman A. Waddington's canalization revisited: developmental stability and evolution. Proc Natl Acad Sci U S A. 2002;6:10528-32.
78. Sinclair BJ, Gibbs AG, Roberts SP. Gene transcription during exposure to, and recovery from, cold and desiccation stress in *Drosophila melanogaster*. Insect Mol Biol. 2007;16:435-43.
79. Stephan W, Li H. The recent demographic and adaptive history of *Drosophila melanogaster*. Heredity. 2007;177:469-80.

80. Stetina T, Kostal V, Korbelova J. The Role of Inducible Hsp70, and Other Heat Shock Proteins, in Adaptive Complex of Cold Tolerance of the Fruit Fly (*Drosophila melanogaster*). PLoS ONE. 2015;10:e0128976.
81. St Pierre SE, Ponting L, Stefancsik R, McQuilton P; FlyBase Consortium. FlyBase 102--advanced approaches to interrogating FlyBase. Nucleic Acids Res. 2014;42:D780-8.
82. Stamnes M. Regulating the actin cytoskeleton during vesicular transport. Curr Opin Cell Biol. 2002;14:428-33.
83. Stephan W, Li H. The recent demographic and adaptive history of *Drosophila melanogaster*. Heredity. 2007;98:65-8.
84. Sternlicht H, Farr GW, Sternlicht ML, Driscoll JK, Willison K, Yaffe MB. The t-complex polypeptide 1 complex is a chaperonin for tubulin and actin in vivo. Proc Natl Acad Sci U S A. 1993;90:9422-6.
85. Svetec N, Werzner A, Wilches R, Pavlidis P, Alvarez-Castro JM, Broman KW, Metzler D, Stephan W. Identification of X-linked quantitative trait loci affecting cold tolerance in *Drosophila melanogaster* and fine mapping by selective sweep analysis. Mol Ecol. 2011;20:530-44.
86. Takayama S, Xie Z, Reed JC. An evolutionary conserved family of *Hsp70*/*Hsc70* molecular chaperone regulators. J Biol Chem. 1999;274:781-6.
87. Teets NM, Peyton JT, Ragland GJ, Colinet H, Renault D, Hahn DA, Denlinger DL. Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. Physiol Genomics. 2012;44:764-77.
88. Telonis-Scott M, Hallas R, McKechnie SW, Wee CW, Hoffmann AA. Selection for cold resistance alters gene transcript levels in *Drosophila melanogaster*. J Insect Physiol. 2009;55:549-55.
89. Tian C, Gao B, Rodriguez Mdel C, Lanz-Mendoza H, Ma B, Zhu S. Gene expression, antiparasitic activity, and functional evolution of the drosomycin family. Mol Immunol. 2008; 45:3909-16.

90. Townsend JP, Rand DM. Mitochondrial genome size variation in New World and Old World populations of *Drosophila melanogaster*. Heredity. 2004;93:98-103.
91. Udaka H, Percival-Smith A, Sinclair BJ. Increased abundance of *Frost* mRNA during recovery from cold stress is not essential for cold tolerance in *Drosophila melanogaster*. Insect Mol Biol. 2013;22:541-50.
92. Vaccari T, Rusten TE, Menut L, Nezis IP, Brech A, Stenmark H, Bilder D. Comparative analysis of ESCRT-I, ESCRT-II and ESCRT-III function in *Drosophila* by efficient isolation of ESCRT mutants. J Cell Sci. 2009;122:2413-23.
93. Vermeulen CJ, Sørensen P, Kirilova Gagalova K, Loeschcke V. Transcriptomic analysis of inbreeding depression in cold-sensitive *Drosophila melanogaster* shows upregulation of the immune response. J Evol Biol. 2013;26:1890-902.
94. Voigt S, Laurent S, Litovchenko M, Stephan W. Positive Selection at the *Polyhomeotic* Locus Led to Decreased Thermosensitivity of Gene Expression in Temperate Drosophila melanogaster. Genetics. 2015;200:591-9.
95. von Heckel K, Stephan W, Hutter S. Canalization of gene expression is a major signature of regulatory cold adaptation in temperate *Drosophila melanogaster*. BMC Genomics. 2016;17:574.
96. Waddington CH. Canalization of development and the inheritance of acquired characters. Nature. 1942;150:563-5.
97. Wagner GP, Kin K, Lynch VJ. Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples. Theory Biosci. 2012;131:281-5.
98. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet. 2009;10:57-63.
99. Whitehead A, Crawford DL. Variation within and among species in gene expression: raw material for evolution. Mol Ecol. 2006;15:1197-211.

100. Wilches R, Voigt S, Duchen P, Laurent S, Stephan W. Fine-mapping and selective sweep analysis of QTL for cold tolerance in *Drosophila melanogaster*. G3 (Bethesda). 2014;4:1635-45.
101. Wray GA, Hahn MW, Abouheif E, Balhoff JP, Pizer M, Rockman MV, Romano LA. The evolution of transcriptional regulation in eukaryotes. Mol Biol Evol. 2003;20:1377-419.
102. Wray GA. The evolutionary significance of *cis*-regulatory mutations. Nat Rev Genet. 2007;8:206-16.
103. Wurmser F, Mary-Huard T, Daudin JJ, Joly D, Montchamp-Moreau C. Variation of gene expression associated with colonisation of an anthropized environment: comparison between African and European populations of *Drosophila simulans*. PLoS One. 2013;8:e79750.
104. Zhang J, Marshall KE, Westwood JT, Clark MS, Sinclair BJ. Divergent transcriptomic responses to repeated and single cold exposures in *Drosophila melanogaster*. J Exp Biol. 2011;214:4021-29.
105. Zhao L, Wit J, Svetec N, Begun DJ. Parallel gene expression differences between low and high latitude populations of *Drosophila melanogaster* and *Drosophila simulans*. PLoS Genet. 2015;11:e1005184.