Proteomic data reveal a physiological basis for costs and benefits associated with thermal acclimation

Kristensen, Torsten Nygaard; Kjeldal, Henrik; Schou, Mads Fristrup; Nielsen, Jeppe Lund

Published in:
Journal of Experimental Biology

DOI (link to publication from Publisher):
10.1242/jeb.132696

Publication date:
2016

Document Version
Publisher's PDF, also known as Version of record

© 2016. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2016) 219, 969-976 doi:10.1242/jeb.132696

RESEARCH ARTICLE

Proteomic data reveal a physiological basis for costs and benefits associated with thermal acclimation

Torsten N. Kristensen[1,*], Henrik Kjeldal[1], Mads F. Schou[2] and Jeppe Lund Nielsen[1]

ABSTRACT

Physiological adaptation through acclimation is one way to cope with temperature changes. Biochemical studies on acclimation responses in ectotherms have so far mainly investigated consequences of short-term acclimation at the adult stage and focussed on adaptive responses. Here, we assessed the consequences of rearing *Drosophila melanogaster* at low (12°C), benign (25°C) and high (31°C) temperatures. We assessed cold and heat tolerance and obtained detailed proteomic profiles of flies from the three temperatures. The proteomic profiles provided a holistic understanding of the underlying biology associated with both adaptive and non-adaptive temperature responses. Results show strong benefits and costs across tolerances: rearing at low temperature increased adult cold tolerance and decreased adult heat tolerance and vice versa with development at high temperatures. In the proteomic analysis, we were able to identify and quantify a large number of proteins compared with previous studies on ectotherms (1440 proteins across all replicates and rearing regimes), enabling us to extend the proteomic approach using enrichment analyses. This gave us detailed information on individual proteins, as well as pathways affected by rearing temperature, pinpointing potential mechanisms responsible for the strong costs and benefits of rearing temperature on functional phenotypes. Several well-known heat shock proteins, as well as proteins not previously associated with thermal stress, were among the differentially expressed proteins. Upregulation of proteasome proteins was found to be an important adaptive process at high-stress rearing temperatures, and occurs at the expense of downregulation of basal metabolic functions.

KEY WORDS: Acclimation, Heat and cold stress, Heat shock proteins, Metabolism, Proteasome, Proteomics

INTRODUCTION

Climate change has raised a great deal of interest in how animals and plants can adapt to expected future climates (Araújo et al., 2013; Chown et al., 2010). Numerous studies have suggested that changes could occur so fast that many species will not be able to adapt through evolutionary changes (Araújo et al., 2013; Hoffmann et al., 2013; Kristensen et al., 2015). Plastic responses, e.g. through physiological adaptation within the lifetime of an individual, is one alternative way to cope with extreme and fluctuating temperatures (Angiletta, 2009; Chown and Terblanche, 2006; Hoffmann and Parsons, 1997; Hoffmann et al., 2003). Studies on ectotherms investigating thermal acclimation and the physiological changes induced in response to acclimation typically do so by exposing adult organisms to short-term cold or hot temperatures and subsequently studying the consequences on thermal and molecular phenotypes (Overgaard et al., 2005; Sørensen et al., 2005; Malmendal et al., 2006; Teranishi and Stillman, 2007; Nota et al., 2010; Teets et al., 2012; Colinet et al., 2013; Jansen et al., 2013; Wei et al., 2015). Such studies have provided novel insights into the molecular aspects of acclimation responses and have added important components to our knowledge on associations between the genotype, the molecular phenotype and thermal resistance. The molecular fingerprints of short-term exposure of adult ectotherms to variable and sometimes stressful temperatures are important if we are to understand the background for thermal acclimation responses. However, in their natural habitats, *Drosophila melanogaster* and other ectotherms are exposed to thermal stress for extended periods e.g. during cold winters or hot summers (Izequierdo, 1991; Rodríguez-Trelles et al., 2013; Schou et al., 2015), making the molecular consequences of long-term exposure of different life stages highly ecologically relevant.

Analysis of the molecular response to temperature changes can be completed by profiling of the functional proteins and can thus provide insight into the regulation of biochemical pathways governing the functional phenotype (Feder and Walser, 2005). One of the first proteomic studies investigating thermal acclimation responses was the pioneering work on the Antarctic archaeonin *Methanococcoides burtonii* (Goodchild et al., 2004). Since then, technologies have been developing fast and state of the art methods now enable identification and quantification of sometimes thousands of proteins in organisms such as *Escherichia coli* (Soufi et al., 2015). In spite of this, studies on thermal acclimation responses in arthropods have rarely identified more than a few hundred proteins (Colinet et al., 2007, 2013; Pedersen et al., 2010; Wei et al., 2015), making investigation of complex physiological changes in response to thermal acclimation difficult.

Here, we expose *D. melanogaster* to three different thermal regimes (12, 25 or 31°C) throughout development from egg to adult and at the first 2-3 days of their adult life, and use label-free quantitative shotgun proteomics on flies from the different thermal regimes. This approach enables us to identify and quantify a large number of differentially expressed proteins affected by the different long-term temperature exposures. Long-term acclimation responses have received little attention in previous proteomic studies, and with the possibility of identifying a large number of proteins, we can take previous proteomic analyses of thermal acclimation to the next level by performing enrichment analyses and thereby identify complex physiological changes. Results from the proteomic analysis were linked to functional phenotypes obtained on flies from the three

[1]Department of Chemistry and Bioscience, Aalborg University, Aalborg E DK-9220, Denmark. [2]Department of Bioscience, Aarhus University, Aarhus C DK-8000, Denmark.

*Author for correspondence (tnk@bio.aau.dk)

Received 30 September 2015; Accepted 15 January 2016

Journal of Experimental Biology (2016) 219, 969-976 doi:10.1242/jeb.132696

temperatures, enabling us to discuss biochemical causes of costs and benefits associated with thermal acclimation.

MATERIALS AND METHODS

Developmental acclimation

The laboratory population used in this study was established from 589 inseminated females caught in Denmark in 2010 (for details, see Schou et al., 2014). Prior to the experiment, the population was maintained at 20°C at 12 h light:12 h dark photoperiod and reared with a standard *Drosophila* medium composed of yeast, oatmeal, sugar and agar. The parental flies used for egg production were density controlled during development (~40 eggs per vial with 7 ml medium). When the parental flies were 4 days of age, they were transferred to bottles with the standard *Drosophila* medium but with a high agar concentration (3%). The following day, eggs were washed off the surface of the medium and distributed into vials in groups of exactly 40 eggs. This methodology was used to ensure random grouping of eggs into vials (Schou, 2013). Vials were allocated to each of three developmental temperatures: 12°C (12 vials), 25°C (8 vials) or 31°C (10 vials). All developmental regimes had a 12 h light:12 h dark photoperiod. On the day of emergence, flies were anaesthetized with CO_2 and counted to obtain estimates of egg-to-adult viability and developmental time. Then, flies were separated into sexes, females were discarded, and males, which were used for proteomics and for thermal resistance measurements, were relocated to the temperature at which they emerged.

Thermal tolerance assays

For each developmental temperature, 20 male flies 2–3 days old (60±12 h) were tested for their critical thermal minimum (CT_{min}) and their critical thermal maximum (CT_{max}) (Overgaard et al., 2012). In these assays, flies were placed individually into sealed 6 ml glass vials and submerged in a water bath at 20°C. When assessing CT_{max}, the bath contained water, in which the temperature was increased at a rate of 0.1°C min^{-1}. Conversely, when assessing CT_{min}, the bath contained a mixture of ethylene glycol and water (1:1, v:v), in which the temperature was decreased at a rate of 0.1°C min^{-1}. Once a fly was immobile when provoked (with a flashlight or gently knocking on the vials with a stick), the temperature of the hot or cold bath was noted as the upper thermal limit (CT_{max}) or lower thermal limit (CT_{min}), respectively.

Data analysis: CT_{min}, CT_{max}, developmental time and egg-to-adult viability data

To investigate the effect of average temperature during development on CT_{max} and CT_{min}, we constructed linear models with temperature as a continuous variable and performed *F*-tests. There were no violations of the assumptions of normality of residuals and homogeneity of variances. Egg-to-adult viability was modelled with a logistic regression, and because *D. melanogaster* has a quadratic performance curve for this trait (e.g. Schou et al., 2015), temperature was modelled as such. We observed overdispersion in the model and corrected the standard errors using a quasi-generalized linear model (Zuur et al., 2009). Developmental time exponentially decreases with increasing temperature (David et al., 1983) and was modelled using a Poisson generalized linear mixed model with a log link and with 'vial' as a random effect. We detected underdispersion in the model, but found it appropriate not to perform any corrections (Zuur et al., 2009). *P*-values for the effect of temperature on egg-to-adult viability and developmental time were obtained by likelihood ratio tests. All statistical analyses were performed in R (v3.2.2; R Core Team, 2015).

Sample preparation for MS analysis

Three replicate samples with 13 male flies at 2-3 days of age (60±12 h) per sample were obtained from each of the three rearing temperatures (12, 25 and 31°C). Proteins were extracted from each of the 9 samples using an optimized protocol based upon the B-PER reagent (Thermo Scientific; as described by Hansen et al., 2014). Proteins were denatured by heating samples at 95°C for 10 min in SDS-PAGE sample buffer supplemented with dithiothreitol (DTT). After boiling, samples were cooled to room temperature prior to loading them onto a pre-cast 4–15% gradient SDS-gel (BioRad). Samples were separated for 30 min at 160 V. Proteins were then digested in-gel (Shevchenko et al., 2007). Briefly, each gel lane was excised and pieces were then washed, reduced and alkylated prior to being digested with trypsin. Digested peptides were extracted, dried down and finally resuspended in 0.1% (v/v) trifluoroacetic acid (TFA), 2% acetonitrile (v/v). Peptides were desalted using the StageTip protocol (Rappsilber et al., 2007). The tryptic digests were resuspended in 0.1% (v/v) TFA, 2% acetonitrile (v/v).

Mass spectrometry

Tryptic digests were analysed by an automated LC-ESI-MS/MS with an UltiMate 3000 RSLCnano system on-line coupled to a Q Exactive mass spectrometer via a Nanospray Flex ion source (Thermo Fisher). Settings were as previously described (Kjeldal et al., 2014), with the following modifications: samples were loaded at a flow rate of 4 µl min^{-1} and eluted at a constant flow rate of 300 nl min^{-1} in a 120 min linear gradient, ranging from 12% to 40% solvent B (100% acetonitrile). Survey scans were acquired at 400–1200 m/z, and no lock mass was used. The maximum ion injection time was set to 100 ms for MS and 75 ms for MS/MS scans. The automatic gain control for MS and MS/MS were 1E6 and 1E5, respectively and a dynamic exclusion of 30 s was used for minimizing repetitive selection of the same ions.

Data analysis: proteomics data

The raw data was analysed with MaxQuant (v1.5.1.2; Cox and Mann, 2008), applying the label-free quantification (LFQ) feature but otherwise using standard settings (data are available via ProteomeXchange with identifier PXD003755). These standard settings included peptide and protein false discovery rates (FDRs) of 1%, tryptic digestion with two allowed miss cleavages, oxidation of methionine as a variable modification and carboxymethylation of cysteine as a fixed modification. Data were cross-referenced with a database containing all proteins from *D. melanogaster* downloaded from UniProt (accessed 6 January 2015).

The generated proteingroups.txt file was imported into Perseus (v1.5.0.31) (part of the MaxQuant package), LFQ values were log_2-transformed and filtered for contaminants and reverse hits before being subjected to statistical analysis. Differences in the expression levels of single proteins between the three temperature regimes were investigated using one-way analysis of variance (ANOVA) followed by a multiple testing correction using a permutation-based FDR of 0.05. The individual *P*-values between groups were calculated using a Student's *t*-test. Statistically significant *P*-values ($P \leq 0.05$) were corrected for multiple testing using the permutation-based FDR of 0.05. Hierarchical clustering in Perseus (v1.5.0.31) was performed with Pearson correlation distance with average linkage after *Z*-scoring of LFQ values. A multi scatter plot was constructed using the log_2-transformed LFQ values of each protein.

Statistically significant differentially regulated proteins (only proteins repetitively found in at least two out of three replicates were

Journal of Experimental Biology (2016) 219, 969-976 doi:10.1242/jeb.132696

Table 1. Results from statistical analysis of life history and thermal resistance traits

Trait	Estimate	s.e.	d.f.	Test statistic	*P*
CT_{max}	0.156	0.008	1,63	F=365.57	<0.001
CT_{min}	0.375	0.011	1,62	F=1077.52	<0.001
Developmental time	−0.104	0.004	1	χ^2=90.24	<0.001
Egg-to-adult viability	2.211 (−1.181)	0.485 (0.519)	2	χ^2=58.51	<0.001

CT_{max} and CT_{min} were analysed with linear models and *P*-values were obtained from *F*-tests, whereas developmental time and egg-to-adult viability were analysed with generalized linear models in which likelihood ratio tests were used to calculate the *P*-values. The effect of temperature on egg-to-adult viability was modelled as a quadratic curve. The estimate and corresponding standard errors of the second-degree term are given in parentheses. Degrees of freedom refer to the numbers of degrees of freedom used with the corresponding test statistic.

included in the analyses) were uploaded to DAVID bioinformatics resources (v6.7) (Huang et al., 2009a,b) and functionally annotated. The output from DAVID was visualized in Cytoscape (v3.2) (Shannon et al., 2003) using the EnrichmentMap plugin (v2.0.1.) (Merico et al., 2010) using a *P*-value cut off of 0.005 and a FDR Q-value cut off of 0.05.

RESULTS

Life history and thermal resistance traits

The effects of developmental temperature on the four traits investigated were strong and all highly significant (Table 1). Thermal tolerance traits increased linearly with increasing developmental temperature; however, at very different rates (Table 1; Fig. 1A,B). CT_{min} increased (cold tolerance decreased) with ∼0.5°C per degree increase in developmental temperature, whereas CT_{max} increased (heat tolerance increased) with only 0.2°C per degree increase in developmental temperature (Table 1). Development from egg to the adult stage at 12°C lasted 50.4±1.6 days, whereas development at 25 and 31°C were faster and lasted 8.9±0.1 and 7.9±0.1 days, respectively (Fig. 1C). Egg-to-adult viability at 12 and 31°C was 66.6% and 92.9%, of the viability at 25°C, respectively (Fig. 1D).

Proteomic analysis of *D. melanogaster*

From the proteomic analysis, a total of 1440 proteins could be quantified from *D. melanogaster* developed and reared at 12°C (1066), 25°C (963) and 31°C (1292), respectively (for full protein lists, see Table S1). Replicates (n=3) within each temperature correlated well despite a modest overlap (Fig. 2 and Fig. 3A–C, Fig. S1). Approximately 54% (789) of all the quantified proteins were shared between the three different rearing regimes (Fig. 3A). Overall, only a small overlap (19 proteins) of the differentially expressed proteins was observed between the three contrasts (25°C vs 12°C, 31°C vs 25°C, and 31°C vs 13°C; Fig. 3B). With increasing temperature, both the number of identifications and number of differentially expressed proteins increased. Contrasting flies reared at 12°C and 25°C revealed 48 proteins (minimum no. unique peptides ≥1) that were differentially regulated (FDR-corrected P≤0.05; Fig. 3B). At 25°C vs 31°C, 132 proteins were differentially regulated (FDR-corrected P≤0.05) and, finally, when

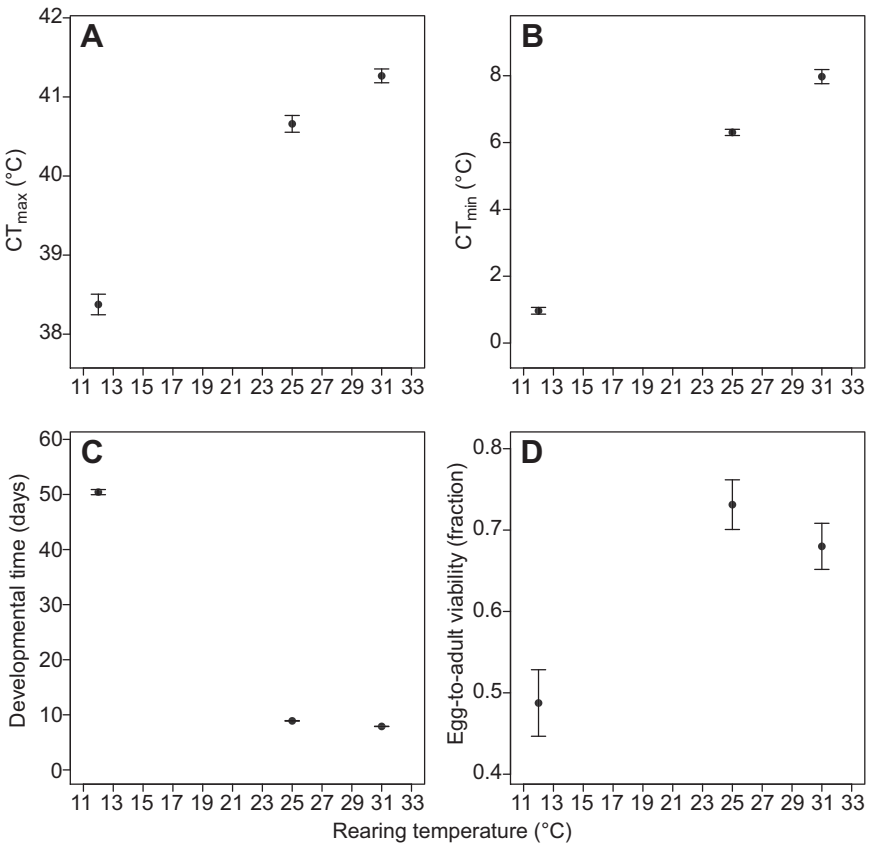

Fig. 1. Thermal resistance of male flies developed and reared at the three developmental temperatures and life history traits assessed in the three thermal environments. (A,B) Critical thermal maximum and minimum for male flies exposed to 12, 25 or 31°C during development and the first 2 days of adult life (until time of testing). (C) Developmental time of males from the egg to the adult stage in days across vials at the three developmental temperatures. (D) Egg-to-adult viability (fraction of eggs developing into an adult fly) across vials at the three developmental temperatures. All values are means±s.e.m.

Journal of Experimental Biology (2016) 219, 969-976 doi:10.1242/jeb.132696

comparing 12°C and 31°C, 223 proteins were differently regulated (FDR-corrected $P \leq 0.05$; Fig. 3B).

The proteomic data was subjected to a functional annotation enrichment analysis in order to identify biological processes, molecular functions and cellular components that were statistically enriched ($P \leq 0.005$, FDR $Q \leq 0.05$) in response to temperature change (Fig. 4). Among the proteins that were differentially upregulated when increasing the temperature from 12°C to 25°C (38 proteins), several functional clusters related to general metabolism/catabolism, energy production, structural components and proteins related to specific enzyme families (hydrolases and oxidoreductases) were enriched (Fig. 4). Proteins that were downregulated in flies from 25°C compared with flies from 12°C (10 proteins), did not enrich for any statistical significant functional cluster (i.e. $P \geq 0.005$, FDR $Q \geq 0.05$). The differentially downregulated proteins included a heat shock protein (P29844) and a CAMP-dependent kinase (P12370), involved in biological processes related to behavioural responses to stimuli (GO:0007610) and adult behaviour (GO:0001654), respectively. Complete protein and GO annotation lists, as well as functional enrichment analysis can be found in Table S1.

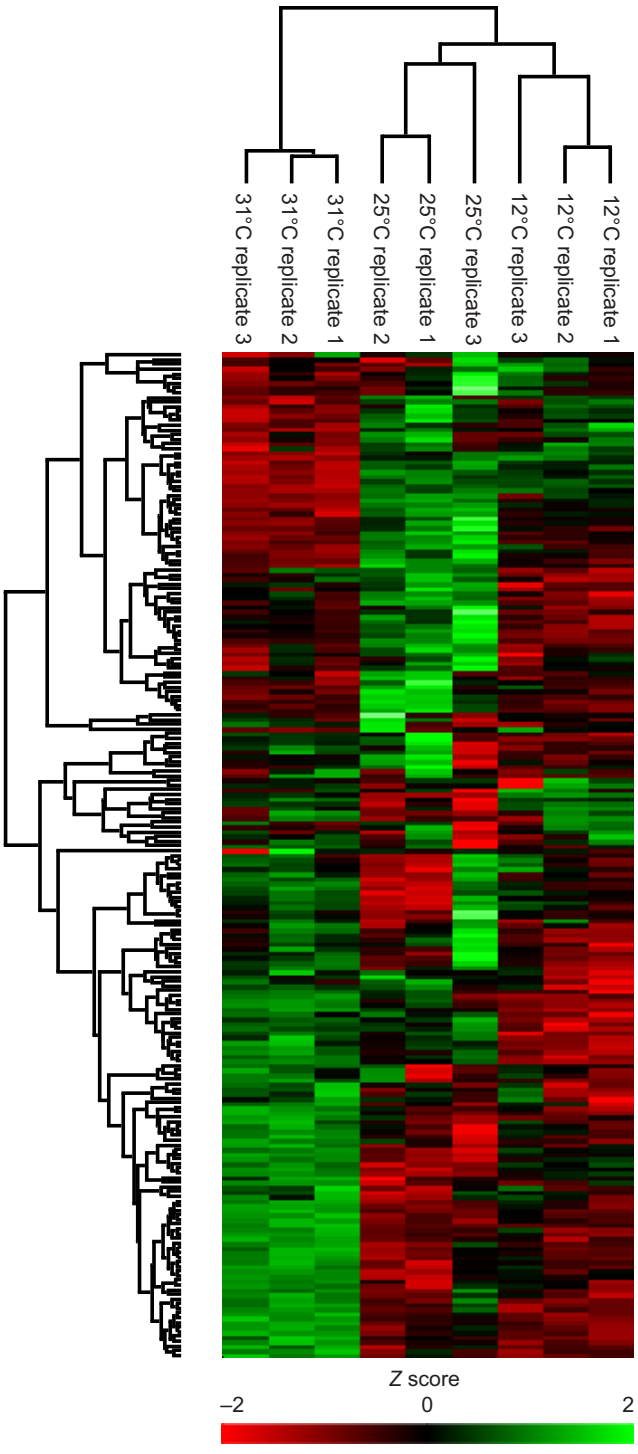

Fig. 2. Hierarchical clustering of proteins using Pearson correlation distance with average linkage based on log_2-transformed LFQ values after *Z*-scoring of differentially expressed proteins across all rearing temperatures and replicates. A total of 1440 proteins were quantified from flies developed and reared at 12°C (1066), 25°C (963) and 31°C (1292), respectively (for full protein lists, see Table S1). Replicates (n=3) within each temperature correlate well despite a modest overlap.

Proteins differentially upregulated at 31°C compared with 25°C (68 proteins), were functionally enriched for a major cluster consisting of proteins related to the proteasome and a minor cluster consisting of phosphoproteins (Fig. 4). The differentially downregulated proteins (56 proteins) formed several clusters functionally related to carbohydrate metabolism/catabolism, energy production, cofactor binding and coenzyme production (pyridoxal phosphate) (Fig. 4).

Contrasting flies developed and reared at 12°C and 31°C, respectively, revealed that the increase in temperature resulted in the differential upregulation of proteins (185 proteins) related to the proteasome, energy production, chemical and cellular homeostasis production of precursor metabolites, and calcium binding proteins (Fig. 4). The differentially downregulated proteins (38 proteins) were mainly related to cofactor binding (Fig. 4).

Several proteins were unique (only identified and quantified) in flies from one of the three temperatures, 12°C (5 proteins), 25°C (28 proteins) and 31°C (123 proteins). Statistically significant enrichment clusters ($P \leq 0.005$, FDR $Q \leq 0.05$) could only be formed for proteins unique to 31°C, revealing five functional clusters comprising proteins related to the proteasome, cytoplasm, hydrolases, thiol proteases and ribonucleoproteins (Fig. S2).

An enrichment analysis was also performed on proteins that were shared between the three different temperatures and for which no statistical significant change in abundance was observed (460 proteins). This enrichment analysis of shared functionality between the three temperatures revealed a multitude of functional clusters most notably clusters related to common metabolic processes, including carbohydrate metabolism, oxidation/reduction reactions and processes related to structural components (Fig. 5). A cluster of several stress-related proteins was also enriched among the proteins for which the abundance was not modulated by changing the developmental and rearing temperature (Fig. 5).

DISCUSSION

In this study, we investigated proteomic profiles, coupled with thermal resistance and life history traits, of *D. melanogaster* flies developed and reared at cold, benign and hot temperatures. Not surprisingly egg-to-adult viability and developmental time were affected by developmental temperature. Development was slower at lower temperatures and egg-to-adult viability was highest at benign temperatures, which is in accordance with well-established results (David and Clavel, 1969; David et al., 1983). Furthermore, we showed that flies reared at 12°C had a CT_{min} that was 5.3 and 7.0°C lower than flies reared at 25 and 31°C, respectively (Fig. 1).

Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 969-976 doi:10.1242/jeb.132696

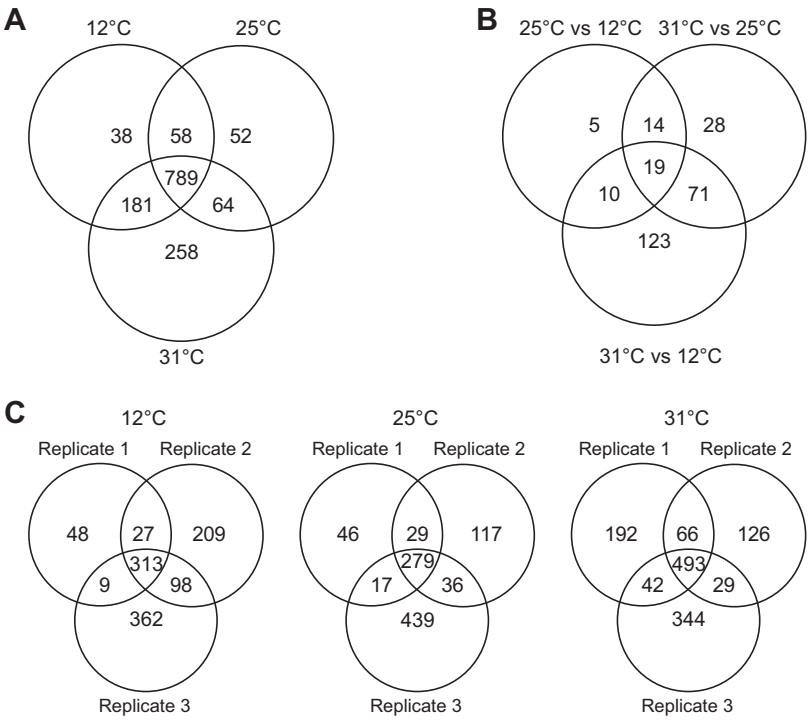

Fig. 3. Venn diagrams to compare all quantified proteins in flies developed and raised at three different temperatures (12°C, 25°C, 31°C). (A) Venn diagram of all identified and quantified proteins across all rearing regimes and all replicates (n=3). A total of 1440 proteins was quantified across all replicates and rearing regimes. (B) Number of unique and overlapping differentially expressed proteins in comparisons of all temperatures. Proteins differentially regulated in all three temperature comparisons are: Q9W3Z3, O97477, X2JF40, Q9V5C6, P61209, Q8IGX3, F0JAL7, P07486, M9NEP1, O01666, Q9W1G0, Q8SXQ1, Q9VQ29, Q9W2X6, Q9W0J9, Q9VKX2, Q9VFN5, A1ZA47 and A1Z7Z4. (C) Venn diagram of all uniquely identified and quantified proteins counted for each rearing regime and its corresponding replicates.

By contrast, flies developed at 31°C had a CT_{max} that was 0.6 and 2.9°C higher than flies developed at 25 and 12°C, respectively (Fig. 1). These results are in accordance with other studies showing marked benefits and costs across tolerances with thermal acclimation (Chidawanyika and Terblanche, 2011; Chown and Terblanche, 2006; Kristensen et al., 2008; Loeschcke and Hoffmann, 2007; Schou et al., 2015). Consequences of climate change include higher and more variable temperatures in many parts of the world (IPCC, 2013). Thus the strong trade-off between tolerances observed here and in other studies clearly suggests that thermal fluctuation constitutes a challenge to ectotherms in nature (Kristensen et al., 2008; Schou et al., 2015) and will potentially be even more of a challenge in the predicted future thermal environment.

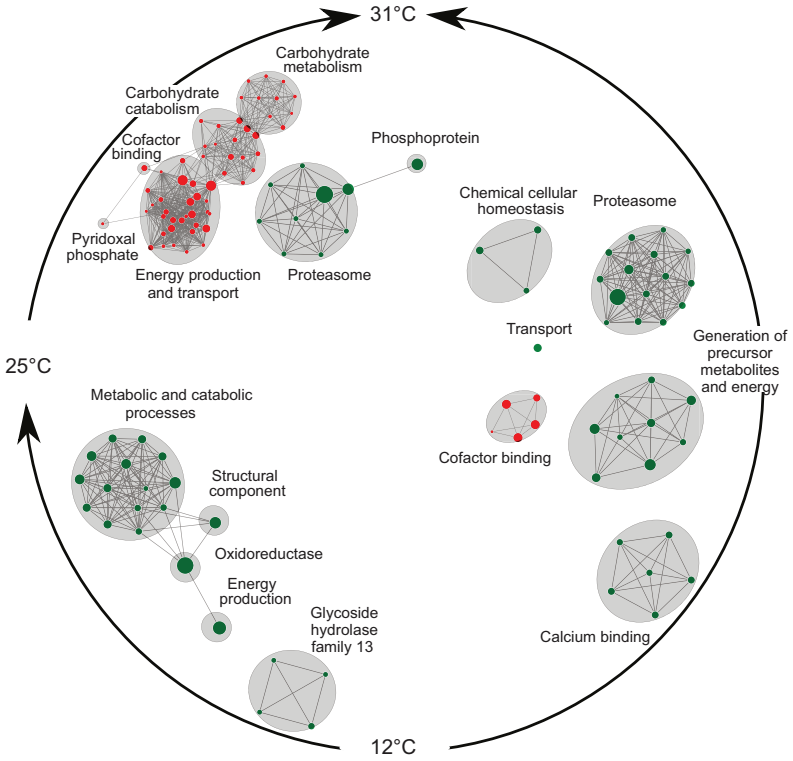

Fig. 4. Annotation clustering of proteins differentially expressed at three different temperatures (12°C, 25°C, 31°C). Only proteins found in at least two out of three replicates were included in the analyses. Green clusters represent proteins that are differentially upregulated (FDR-corrected $P \leq 0.05$), whereas red clusters represent differentially downregulated proteins in response to temperature changes. Nodes represent protein sets and edges represent mutual overlap, whereas the size of each node represents the number of proteins in the protein set.

Journal of Experimental Biology (2016) 219, 969-976 doi:10.1242/jeb.132696

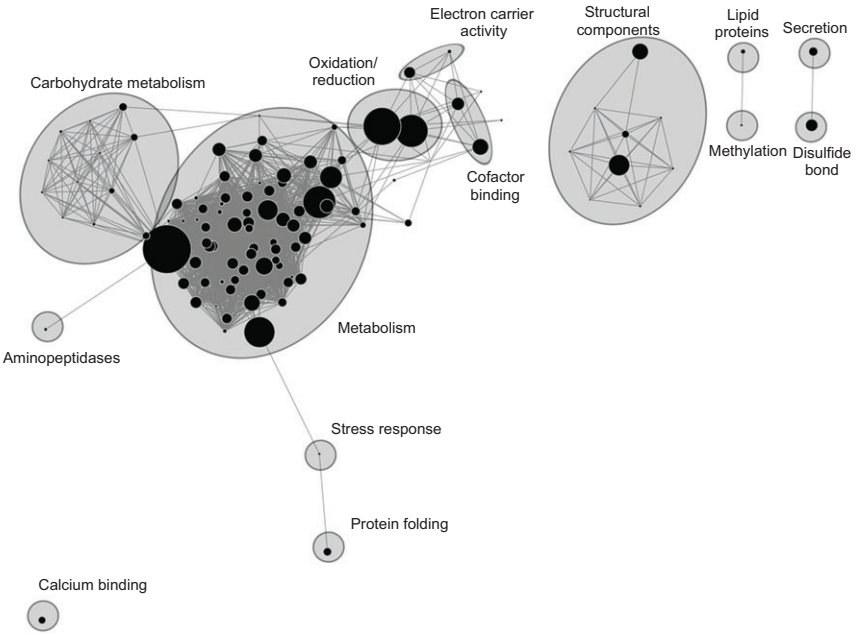

Fig. 5. Annotation clustering of proteins shared between all three rearing regimes (12°C, 25°C, 31°C). Only proteins repetitively found in at least two out of three replicates (a total of 460 proteins) were included in the analyses. Nodes represent protein sets and edges represent mutual overlap, whereas the size of each node represents number of proteins in the protein set.

Our data also provide novel insights into the underlying physiology of the strong acclimation responses observed in the functional phenotypes. Replicates within temperatures clustered neatly and the three rearing temperatures were clearly separated, with flies from 12°C and 25°C having a more similar proteomic profile compared with flies reared at 31°C (Figs 2, 3 and Fig. S1). Thus despite cold acclimation inducing the strongest functional phenotypic response in terms of increased cold resistance, decreased egg-to-adult viability and developmental time (Fig. 1), effects on the proteomic level were small compared with effects of heat acclimation (Figs 2–4). This finding is similar to that of previous studies on the transcriptional and proteomic levels (Colinet et al., 2013; Overgaard et al., 2014) and suggests a non-linear relationship between cellular acclimation responses and functional phenotypic responses.

Contrasting results in flies reared at 25°C and 31°C

The proteomic profile of flies reared at 31°C compared with flies reared at 25°C was characterized by downregulation of proteins and multiple clusters associated with carbohydrate catabolism, carbohydrate metabolism, energy production and transport, and by upregulation of the proteasome (Fig. 4, Table S1). Thus, the data suggest that rearing at high and stressful temperatures leads to reduced conversion of carbohydrates and therefore probably lower energy production (such as ATP). In contrast to metabolic proteins, proteasome proteins involved in repair and degradation of oxidative or structural (misfolding) damaged proteins are upregulated in flies reared at 31°C compared with flies reared at 25°C (Fig. 4, Table S1). Among the upregulated proteins are several heat shock proteins known for being part of the well-described heat shock response (Colinet et al., 2013; Kültz, 2005; Sørensen et al., 2003), as well as proteins not previously associated with thermal stress responses (Table S1).

Despite the observed downregulation of proteins involved in key metabolic processes, basal metabolic rate increases in accordance with expectations for Q10 when exposing *D. melanogaster* to temperatures within the range investigated here (Jensen et al., 2014). Upregulation of the proteasome, including molecular chaperones is energetically costly (Kültz, 2005; Sørensen et al., 2003). Specifically, upregulation of heat shock proteins (Hsps) has been shown to be associated with downregulation of many other proteins, partly because it is energetically demanding (Sørensen et al., 2003). Thus, flies reared at high and stressful temperatures are likely to be in a state of energetic deficiency, i.e. there is a mismatch between supply and demand (Schulte, 2015). Whether downregulation of proteins involved in key metabolic processes in flies exposed to 31°C is part of an adaptive cellular stress response or a maladaptive consequence of exposure to stressful high temperatures is unresolved. It is, however, well known that induction of Hsps has a negative impact on development and survival, cell division and energy balance in *Drosophila* (Feder et al., 1992; Krebs and Feder, 1997; Krebs and Loeschcke, 1994; Silbermann and Tatar, 2000). We therefore propose that upregulation of proteasome proteins is an important adaptive process at stressful high temperatures, which increases heat tolerance by ridding cells of potentially harmful substances that arise as a result of misfolding of proteins and post-transcriptional and post-translational changes due to thermal damage. This occurs at the expense of downregulation of basal metabolic functions. The explanation fits in well with observations that longevity and reproduction decrease sharply at temperatures above 29–30°C in *D. melanogaster* (David et al., 1983; Linford et al., 2013; Miquel et al., 1976; Schnebel and Grossfield, 1984).

Previous studies have proposed a general adaptive stress response in which the same mechanisms are invoked across different stressors (e.g. Kültz, 2005). Our observation that very few proteins overlap when differentially expressed proteins are compared in the 25°C vs 31°C, 31°C vs 12°C and 25°C vs 12°C groups (Fig. 3B) provide evidence against such common physiological plastic responses in cold and hot thermal environments (see also Bubliy et al., 2012). Despite this general observation, we did identify a small number of proteins that were differentially expressed in all temperature comparisons (Fig. 3B). These proteins are likely to be under strong selection in a more variable climate in which the organism is faced with interchanging heat and cold stress, and are thus candidate genes of strong evolutionary interest that should be investigated more thoroughly in future studies. This group of proteins included proteins related to signal transduction (P61209), energy (O01666, Q9VQ29 and Q9W2X6) and carbohydrate metabolism (P07486, Q9VKX2, Q9W1G0, Q9W3Z3 and X2JF40), as well as proteins related to

Journal of Experimental Biology (2016) 219, 969-976 doi:10.1242/jeb.132696

various stress responses, including salvage pathways (Q8SXQ1 and Q8IGX3), protein folding (Q9VFN5) and proteasomal degradation (Q9V5C6).

Contrasting results in flies reared at 12°C and 31°C

Comparing the proteomic profiles of flies reared at 12°C and 31°C revealed that multiple clusters associated with cellular homeostasis, the proteasome, generation of precursor metabolites and energy, and calcium binding were upregulated in flies reared at 31°C (Fig. 4). By contrast, some proteins involved in co-factor binding were downregulated in flies reared at 31°C compared with 12°C (Fig. 4). The majority of the significant clusters in this contrast are thus upregulated at 31°C (Figs 2 and 4, Table S1). This is unlike the 25°C and 31°C comparison, where most clusters were downregulated at 31°C (Fig. 4). However, common to the two contrasts is an upregulation of the proteasome and a downregulation of cofactor binding at 31°C (Fig. 4). In temperate climates, *D. melanogaster* is known to survive winters in the adult state, where they are often exposed to temperatures below 10°C for several months (Izequierdo, 1991; Schou et al., 2015), resulting in a longer lifespan (Miquel et al., 1976). This suggests fundamental differences in the type of stress induced by 12°C and 31°C, which is supported by the proteomic data (Figs 2–4 and Table S1). For example, flies at 12°C are unlikely to suffer from protein damage, whereby the advantage of inducing the proteasome stress response is small (Fig. 4, Table S1). Other significant clusters and proteins upregulated in flies developed at 31°C compared with 12°C are involved in maintaining cellular homeostasis, generation of energy and calcium binding (Fig. 4). The changes in such processes are likely to be related to the increased metabolism in flies reared at 31°C compared with 12°C (Jensen et al., 2014; Schulte, 2015).

Contrasting results in flies reared at 12°C and 25°C

Comparison of proteomic profiles of flies reared at 12°C and 25°C revealed upregulation of all identified clusters in flies from 25°C, including clusters with proteins involved in major metabolic processes (Fig. 4), especially proteins involved in glycoside hydrolase, carbohydrate metabolism and catabolism, as well as structural components, oxidoreductase and energy production (Fig. 4). At 12°C, we detected few upregulated proteins (and no clusters) and none of these was part of the classical stress response (Kültz, 2005; Sørensen et al., 2003). The number of proteins uniquely expressed at 12°C was also low (Fig. 3A, Table S1). This suggests that although development at 12°C is stressful because only a fraction of the flies reach the adult stage, the adult life stage in itself is not under the same degree of stress. Thus the proteomic effect of rearing at low temperatures is minor and the functional phenotypic effects in terms of increased cold resistance, decreased heat resistance and increased developmental time are strong (Fig. 1). The lower basal metabolic rate in ectotherms at low compared with higher temperatures (Jensen et al., 2014; Schulte, 2015) may be important for understanding the costs and benefits associated with cold acclimation (Schulte, 2015). Flies acclimated to low temperatures have a generally low metabolic rate, which might provide these flies with benefits at low temperatures. Transferring the same flies to high temperatures will result in strong costs in survival as a result of reduced heat resistance (and vice versa with flies reared at high temperatures). Distinguishing cause from effect is a challenge that we cannot solve here, but our data support the idea that metabolic rate is key to understanding the strong costs and benefits typically observed in studies on thermal acclimation (Angiletta, 2009; Chidawanyika and Terblanche, 2011; Kristensen et al., 2008; Schou et al., 2015; Schulte, 2015).

Conclusion

Three lines of evidence in this study suggest that the physiological acclimation responses to cold and hot temperatures are clearly distinct (Bubliy et al., 2012): (1) the proteomic differentiation between flies from 12°C and 31°C; (2) the very low overlap observed when comparing differentially expressed proteins at 25°C vs 31°C with those differentially expressed at 25°C vs 12°C; and (3) the fact that 258 unique proteins are identified in flies reared at 31°C and only 38 unique proteins are identified in flies reared at 12°C. This provides a physiological explanation for the costs observed when exposing ectotherms to temperatures that are different to acclimation temperatures (Chidawanyika and Terblanche, 2011; Chown and Terblanche, 2006; Kristensen et al., 2008; Loeschcke and Hoffmann, 2007; Sørensen et al., 2013), as well as the trade-off between CT_{min} and CT_{max} observed in this study. More specifically, we found evidence that upregulation of proteasome proteins is an important adaptive process at stressful high temperatures, but this is at the expense of downregulation of basal metabolic functions. Thus, the interplay between metabolism or metabolic rate and the proteasome is key to the adaptive response across the thermal range.

Acknowledgements

We are grateful to Volker Loeschcke for his unfailing encouragement and support and to Doth Andersen and Helle Blendstrup for technical assistance.

Competing interests

The authors declare no competing or financial interests.

Author contributions

M.F.S. and T.N.K. conceived and designed the experiments. M.F.S., H.K. and T.N.K. performed the experiments. All authors contributed to analysing the data and writing the paper.

Funding

This research was funded by Aalborg University and the Danish Natural Science Research Council with a Sapere aude stipend to T.N.K. [DFF–4002-00036].

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD003755 (http://www.ebi.ac.uk/pride/archive/projects/PXD003755).

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.132696/-/DC1

References

Angiletta, M. J. (2009). *Thermal Adaptation: A Theoretical and Empirical Analysis*. New York: Oxford Univ. Press.

Araújo, M. B., Ferri-Yáñez, F., Bozinovic, F., Marquet, P. A., Valladares, F. and Chown, S. L. (2013). Heat freezes niche evolution. *Ecol. Lett.* **16**, 1206-1219.

Bubliy, O. A., Kristensen, T. N., Kellermann, V. and Loeschcke, V. (2012). Plastic responses to four environmental stresses and cross-resistance in a laboratory population of *Drosophila melanogaster*. *Funct. Ecol.* **26**, 245-253.

Chidawanyika, F. and Terblanche, J. S. (2011). Costs and benefits of thermal acclimation for codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae): implications for pest control and the sterile insect release programme. *Evol. Appl.* **4**, 534-544.

Chown, S. L. and Terblanche, J. S. (2006). Physiological diversity in insects: ecological and evolutionary contexts. *Adv. Insect Physiol.* **33**, 50-152.

Chown, S. L., Hoffmann, A. A., Kristensen, T. N., Angilletta, M. J., Stenseth, N. C. and Pertoldi, C. (2010). Adapting to climate change: a perspective from evolutionary physiology. *Clim. Res.* **43**, 3-15.

Colinet, H., Nguyen, T. T. A., Cloutier, C., Michaud, D. and Hance, T. (2007). Proteomic profiling of a parasitic wasp exposed to constant and fluctuating cold exposure. *Insect Biochem. Mol. Biol.* **37**, 1177-1188.

Colinet, H., Overgaard, J., Com, E. and Sørensen, J. G. (2013). Proteomic profiling of thermal acclimation in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* **43**, 352-365.

Cox, J. and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* **26**, 1367-1372.
David, J. R. and Clavel, M. D. (1969). Temperature effect on number hatching percentage and size of eggs laid by *Drosophila melanogaster*. *Ann. la Société Entomol. Fr.* **5**, 161-171.
David, J. R., Allemand, R., Vand Herrewege, J. and Cohet, Y. (1983). Ecophysiology: abiotic factors. In *The Genetics and Biology of Drosophila* (ed. M. Ashburner, H. L. Carson and J. N. Thompson, Jr), pp. 105-170. New York: Acedemic Press.
Feder, M. E. and Walser, J.-C. (2005). The biological limitations of transcriptomics in elucidating stress and stress responses. *J. Evol. Biol.* **18**, 901-910.
Feder, J. H., Rossi, J. M., Solomon, J., Solomon, N. and Lindquist, S. (1992). The consequences of expressing hsp70 in *Drosophila* cells at normal temperatures. *Genes Dev.* **6**, 1402-1413.
Goodchild, A., Saunders, N. F. W., Ertan, H., Raftery, M., Guilhaus, M., Curmi, P. M. G. and Cavicchioli, R. (2004). A proteomic determination of cold adaptation in the Antarctic archaeon, *Methanococcoides burtonii*. *Mol. Microbiol.* **53**, 309-321.
Hansen, S. H., Stensballe, A., Nielsen, P. H. and Herbst, F.-A. (2014). Metaproteomics: evaluation of protein extraction from activated sludge. *Proteomics* **14**, 2535-2539.
Hoffmann, A. A. and Parsons, P. A. (1997). *Extreme Environmental Change and Evolution*. Cambridge, UK: Cambridge University Press.
Hoffmann, A. A., Sørensen, J. G. and Loeschcke, V. (2003). Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *J. Therm. Biol.* **28**, 175-216.
Hoffmann, A. A., Chown, S. L. and Clusella-Trullas, S. (2013). Upper thermal limits in terrestrial ectotherms: how constrained are they? *Funct. Ecol.* **27**, 934-949.
Huang, D. W., Sherman, B. T. and Lempicki, R. A. (2009a). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**, 44-57.
Huang, D. W., Sherman, B. T. and Lempicki, R. A. (2009b). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* **37**, 1-13.
IPCC (2013). Summary for policymakers. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (ed. T. F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P. M. Midgley), pp. 1-33. Cambridge: Cambridge University Press.
Izequierdo, J. I. (1991). How does *Drosophila melanogaster* overwinter? *Entomol. Exp. Appl.* **59**, 51-58.
Jansen, M., Vergauwen, L., Vandenbrouck, T., Knapen, D., Dom, N., Spanier, K. I., Cielen, A. and De Meester, L. (2013). Gene expression profiling of three different stressors in the water flea *Daphnia magna*. *Ecotoxicology* **22**, 900-914.
Jensen, P., Overgaard, J., Loeschcke, V., Schou, M. F., Malte, H. and Kristensen, T. N. (2014). Inbreeding effects on standard metabolic rate investigated at cold, benign and hot temperatures in *Drosophila melanogaster*. *J. Insect Physiol.* **62**, 11-20.
Kjeldal, H., Pell, L., Pommerening-Röser, A. and Nielsen, J. L. (2014). Influence of *p*-cresol on the proteome of the autotrophic nitrifying bacterium *Nitrosomonas eutropha* C91. *Arch. Microbiol.* **196**, 497-511.
Krebs, R. A. and Feder, M. E. (1997). Deleterious consequences of Hsp70 overexpression in *Drosophila melanogaster* larvae. *Cell Stress Chaperones* **2**, 60-71.
Krebs, R. A. and Loeschcke, V. (1994). Costs and benefits of activation of the heat-shock response in *Drosophila melanogaster*. *Funct. Ecol.* **8**, 730-737.
Kristensen, T. N., Hoffmann, A. A., Overgaard, J., Sørensen, J. G., Hallas, R. and Loeschcke, V. (2008). Costs and benefits of cold acclimation in field-released *Drosophila*. *Proc. Natl. Acad. Sci. USA* **105**, 216-221.
Kristensen, T. N., Overgaard, J., Lassen, J., Hoffmann, A. A. and Sgrò, C. (2015). Low evolutionary potential for egg-to-adult viability in *Drosophila melanogaster* at high temperatures. *Evolution* **69**, 803-814.
Kültz, D. (2005). Molecular and evolutionary basis of the cellular stress response. *Annu. Rev. Physiol.* **67**, 225-257.
Linford, N. J., Bilgir, C., Ro, J. and Pletcher, S. D. (2013). Measurement of lifespan in *Drosophila melanogaster*. *J. Vis. Exp.* e50068.
Loeschcke, V. and Hoffmann, A. A. (2007). Consequences of heat hardening on a field fitness component in *Drosophila* depend on environmental temperature. *Am. Nat.* **169**, 175-183.
Malmendal, A., Overgaard, J., Bundy, J. G., Sørensen, J. G., Nielsen, N. C., Loeschcke, V. and Holmstrup, M. (2006). Metabolomic profiling of heat stress: hardening and recovery of homeostasis in *Drosophila*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **291**, R205-R212.
Merico, D., Isserlin, R., Stueker, O., Emili, A. and Bader, G. D. (2010). Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. *PLoS ONE* **5**, e13984.
Miquel, J., Lundgren, P. R., Bensch, K. G. and Atlan, H. (1976). Effects of temperature on the life span, vitality and fine structure of *Drosophila melanogaster*. *Mech. Ageing Dev.* **5**, 347-370.
Nota, B., Van Straalen, N. M., Ylstra, B. and Roelofs, D. (2010). Gene expression microarray analysis of heat stress in the soil invertebrate *Folsomia candida*. *Insect Mol. Biol.* **19**, 315-322.
Overgaard, J., Sørensen, J. G., Petersen, S. O., Loeschcke, V. and Holmstrup, M. (2005). Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. *J. Insect Physiol.* **51**, 1173-1182.
Overgaard, J., Kristensen, T. N. and Sørensen, J. G. (2012). Validity of thermal ramping assays used to assess thermal tolerance in arthropods. *PLoS ONE* **7**, e32758.
Overgaard, J., Sørensen, J. G., Com, E. and Colinet, H. (2014). The rapid cold hardening response of *Drosophila melanogaster*: complex regulation across different levels of biological organization. *J. Insect Physiol.* **62**, 46-53.
Pedersen, K. S., Codrea, M. C., Vermeulen, C. J., Loeschcke, V. and Bendixen, E. (2010). Proteomic characterization of a temperature-sensitive conditional lethal in *Drosophila melanogaster*. *Heredity* **104**, 125-134.
Rappsilber, J., Mann, M. and Ishihama, Y. (2007). Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat. Protoc.* **2**, 1896-1906.
Rodríguez-Trelles, F., Tarrío, R. and Santos, M. (2013). Genome-wide evolutionary response to a heat wave in *Drosophila*. *Biol. Lett.* **9**, 20130228.
Schnebel, E. M. and Grossfield, J. (1984). Mating-temperature range in *Drosophila*. *Evolution* **38**, 1296-1307.
Schou, M. F. (2013). Fast egg collection method greatly improves randomness of egg sampling in *Drosophila melanogaster*. *Fly* **7**, 44-46.
Schou, M. F., Kristensen, T. N., Kellermann, V., Schlötterer, C. and Loeschcke, V. (2014). A *Drosophila* laboratory evolution experiment points to low evolutionary potential under increased temperatures likely to be experienced in the future. *J. Evol. Biol.* **27**, 1859-1868.
Schou, M. F., Loeschcke, V. and Kristensen, T. N. (2015). Strong costs and benefits of winter acclimatization in *Drosophila melanogaster*. *PLoS ONE* **10**, e0130307.
Schulte, P. M. (2015). The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *J. Exp. Biol.* **218**, 1856-1866.
Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., Amin, N., Schwikowski, B. and Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* **13**, 2498-2504.
Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V. and Mann, M. (2007). In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* **1**, 2856-2860.
Silbermann, R. and Tatar, M. (2000). Reproductive costs of heat shock protein in transgenic *Drosophila melanogaster*. *Evolution* **54**, 2038-2045.
Sørensen, J. G., Kristensen, T. N. and Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecol. Lett.* **6**, 1025-1037.
Sørensen, J. G., Nielsen, M. M., Kruhøffer, M., Justesen, J. and Loeschcke, V. (2005). Full genome gene expression analysis of the heat stress response in *Drosophila melanogaster*. *Cell Stress Chaperones* **10**, 312-328.
Sørensen, C. H., Toft, S. and Kristensen, T. N. (2013). Cold-acclimation increases the predatory efficiency of the aphidophagous coccinellid *Adalia bipunctata*. *Biol. Control* **65**, 87-94.
Soufi, B., Krug, K., Harst, A. and Macek, B. (2015). Characterization of the *E. coli* proteome and its modifications during growth and ethanol stress. *Front. Microbiol.* **6**, 103.
Teets, N. M., Peyton, J. T., Ragland, G. J., Colinet, H., Renault, D., Hahn, D. A. and Denlinger, D. L. (2012). Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. *Physiol. Genomics* **44**, 764-777.
Teranishi, K. S. and Stillman, J. H. (2007). A cDNA microarray analysis of the response to heat stress in hepatopancreas tissue of the porcelain crab *Petrolisthes cinctipes*. *Comp. Biochem. Physiol. D Genomics Proteomics* **2**, 53-62.
The Uniprot Consortium (2014). UniProt: a hub for protein information. *Nucleic Acids Res.* **43**, D204-D212.
Vizcaíno, J. A., Csordas, A., del-Toro, N., Dianes, J. A., Griss, J., Lavidas, I., Mayer, G., Perez-Riverol, Y., Reisinger, F., Ternent, T., Xu, Q. W., Wang, R. and Hermjakob, H. (2016). 2016 update of the PRIDE database and related tools. *Nucleic Acids Res.* **44**, D447-D456.
Wei, D., Jia, F.-X., Tian, C.-B., Tian, Y., Smagghe, G., Dou, W. and Wang, J.-J. (2015). Comparative proteomic analysis of *Bactrocera dorsalis* (Hendel) in response to thermal stress. *J. Insect Physiol.* **74**, 16-24.
Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A. and Smith, G. M. (2009). *Mixed Effects Models and Extensions in Ecology with R*. New York: Springer.

Journal of Experimental Biology