Evolutionary Ecology Research, 2012, **14**: 821–837

Inconsistent effects of developmental temperature acclimation on low-temperature performance and metabolism in *Drosophila melanogaster*

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ABSTRACT

Question: Does acclimation to developmental temperature consistently affect metabolism and low-temperature performance when measured in different laboratory and field assays?

Hypothesis: Developmental acclimation reflecting naturally fluctuating thermal conditions consistently increases different components of performance at low temperatures and results in a clearly defined metabolic signature.

Organism: The fruit fly *Drosophila melanogaster* acclimated at different temperatures during development under semi-field and laboratory conditions

Field site: Mt. Rothwell (37°53′22″S, 144°26′25″E) and laboratory facilities at the University of Melbourne, Victoria, Australia.

Methods: Adult females developed under four different constant or fluctuating thermal conditions were tested for their ability to locate food under cool conditions, recovery from a cold shock, or fecundity. Flies from the different acclimation regimes were characterized biochemically using NMR-based metabolomics.

Conclusions: Flies reared at constant benign temperatures were more fecund at all acclimation temperatures. In contrast, flies reared under fluctuating natural or laboratory conditions were more successful in locating food under cool conditions in the field, while constant cool rearing conditions led to high cold resistance. The fluctuating- and low-temperature rearing conditions resulted in a similar metabolic profile, while the 24°C rearing profile was distinct and showed a lack of plasticity. The effects of developmental acclimation on performance are therefore complex and cannot be captured through experimental comparisons of constant environments.

Keywords: beneficial acclimation hypothesis, cold tolerance, fluctuating vs. constant temperatures, life-history traits, metabolomics, plasticity, semi-natural conditions.

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INTRODUCTION

Tolerance to thermal stress is an important environmental factor affecting the distribution and abundance of organisms (Cossins and Bowler, 1987; Hoffmann and Parsons, 1991; Angilletta, 2009). To cope with thermal stress, ectotherms may have high inherent tolerance and also the capacity to respond to temperature variation by adaptive phenotypic plasticity (Levins, 1969; Cossins and Bowler, 1987; Angilletta, 2009). Recent studies on drosophilids have suggested that changes in inherent resistance may be constrained (Mitchell and Hoffmann, 2010; Kellermann *et al.*, 2012a, 2012b), emphasizing the importance of plastic responses for coping with climate changes and climatic variation both now and in the future. Phenotypic plasticity can be defined as the ability of a genotype to change its phenotype in response to environmental changes (Bradshaw, 1965). One form of plasticity is known as thermal acclimation, where organisms adjust their physiology. Such acclimation often leads to improved fitness under thermally extreme conditions; when they are acclimated to warm or cold sub-lethal conditions, they have an increased ability to tolerate extreme hot and cold temperatures, respectively (Levins, 1969; Thomson *et al.*, 2001; Kristensen *et al.*, 2008; Chidawanyika and Terblanche, 2011). This suggests that, under extreme conditions, acclimation can be 'beneficial', unlike in comparisons of performance across less stressful environments, where organisms may not necessarily have the highest fitness under the conditions in which they are acclimated (Leroi *et al.*, 1994; Huey *et al.*, 1999; Woods and Harrison, 2001; Wilson and Franklin, 2002; Chown and Terblanche, 2007).

Although acclimation can increase tolerance of extremes, there may nevertheless be costs, affecting performance under different thermal conditions (Kristensen *et al.*, 2008; Chevin *et al.*, 2010; Basson *et al.*, 2012). Costs and benefits should therefore be taken into account when evaluating the overall effects of acclimation, preferably using ecologically relevant conditions (Deere and Chown, 2006; Zhen *et al.*, 2011; Basson *et al.*, 2012). In this respect, several recent studies have shown that acclimation responses are dependent on both mean temperatures and the variance in temperature (Arias *et al.*, 2010; Terblanche *et al.*, 2010; Niehaus *et al.*, 2012), suggesting that laboratory investigations performed at constant temperatures may not reflect thermal tolerance directly relevant to nature (Deere and Chown, 2006; Terblanche *et al.*, 2010). Moreover, assays of performance typically employed in the laboratory, such as of fecundity and various life-history traits, may underestimate costs under field conditions (Loeschcke and Hoffmann, 2007; Kristensen *et al.*, 2008).

In this study, we tested whether two constant temperatures (17°C and 24°C) and two fluctuating treatments (12–22°C and field conditions) during development had consistent effects on three measures of low-temperature performance in *Drosophila melanogaster*. We first characterized the metabolomic consequences of the four regimes using NMR-based metabolomics. Recently, a range of transcriptomic, proteomic, and metabolomic studies have investigated the molecular and physiological basis of thermal acclimation responses (e.g. Sørensen *et al.*, 2005; Malmendal *et al.*, 2006; Overgaard *et al.*, 2007; Fields *et al.*, 2012), but these have mostly been performed under constant laboratory temperatures. We also considered the way in which the acclimation treatments influenced recovery after a cold stress, fecundity at cool and benign conditions, and the ability of flies to locate resources in nature under cool conditions. The results indicate a complex set of responses where temperature fluctuations affect some traits but not others, and the rank order of beneficial effects changes depending on the measure of performance.

METHODS AND MATERIALS

Origin and maintenance of flies

Drosophila melanogaster flies were derived from a mass-bred population originating from Melbourne (Victoria, Australia, latitude 37°77′S) and maintained in the laboratory on a standard corn, yeast, dextrose, and agar medium at 24 ± 1°C under constant light. The population was established by combining approximately 40 isofemale lines collected in the field in March to April 2008. After pooling the isofemale lines, the population was maintained in high numbers ($N > 500$) for seven generations in the laboratory before the present experiments began.

Eggs from the mass-bred population were collected in the laboratory and were then left to develop in four different developmental acclimation treatments: (1) a constant 24°C and 12 h : 12 h light/dark cycle; (2) a constant 17°C and 12 h : 12 h light/dark cycle; (3) variable temperature with a mean of 17°C (12°C in darkness for 12 h and 22°C with light for 12 h); and (4) natural thermal and light conditions under semi-field conditions with a mean temperature of 15.6°C (minimum and maximum temperatures of 8°C and 27°C, respectively). In the natural thermal acclimation treatment, bottles and vials with developing eggs were positioned in the shade outside Melbourne University in September 2008. In the 17°C variable temperature regime, the lower temperature of 12°C was experienced between 08.00 to 20.00 h. Light regimes followed the same rhythm in all laboratory regimes and flies developing outside were exposed to natural light cycles. Flies emerging from the four developmental treatments were used in multiple experiments assessing field response to bait, laboratory thermal tolerance, fecundity, and metabolite composition (see descriptions below).

Release–capture experiments

To assess field performance at low temperature, we performed a release experiment that evaluates the animal's ability to locate a resource in a natural habitat that is otherwise free of breeding sites.

Flies developing at the four different acclimation regimes were maintained in 600 mL bottles each containing 100 mL of sugar–agar–dead yeast medium with propionic acid and methyl *p*-hydroxybenzoate as preservatives. Eggs to be distributed to the four acclimation regimes were all laid in the laboratory at 24°C within an 8 h period. After this time, adult flies were removed and bottles were distributed among the four acclimation treatments. Density in the bottles was partly controlled by leaving approximately one hundred 5-day-old adults in each bottle to lay eggs for 8 h. This ensured that flies developed at an intermediate density (300–400 offspring per bottle). After emergence, females were sampled (under CO_2 anaesthesia) and transferred to new bottles with fresh medium and held at the respective acclimation temperature for 5 days until we performed the releases. Since developmental time differs between acclimation regimes, eggs were produced at different times so that adult flies emerged at the same time (pilot experiments indicated the approximate developmental time under the different temperature conditions).

On the day before the releases, 5- to 6-day-old female flies from the four acclimation regimes were transferred to vials each with 5 mL of medium (50 flies per vial) and kept at the respective developmental temperature until transport to the release site. We compared

Table 1. Release number, average capture (%), and temperature recordings during the experiment

		Temperature (°C)						
Release number	Average capture (%)	At the time of release	Average during night (08.00–20.00 h)	1. Capture (10.00 h)	2. Capture (12.00 h)	3. Capture (14.00 h)	4. Capture (16.00 h)	5. Capture (18.00 h)
1	22.17	10.6	8.9	11.0	15.2	17.3	18.8	14.0
2	19.53	11.4	9.6	11.7	16.2	18.3	17.3	14.5
3	19.83	10.8	9.3	12.3	16.8	19.2	16.2	15.2

Note: 750 female flies were released from each acclimation regime in each of the 3 releases.

female flies from the four acclimation regimes in three separate releases at cool temperatures. Flies from the four acclimation regimes were positioned in the field in vials in the evening after the temperature had dropped below 12°C. The following morning, at around 09.00 h, we released flies. At 10.00 h we started to capture flies at baits positioned in the vicinity of the release site. Temperatures at which we captured flies during the night and the next day are shown in Table 1.

Flies were transported by car to the release sites in insulated styrofoam boxes kept at 22–24°C (transportation took approximately 2 h). Just before release, groups of approximately 100 flies were transferred into vials with 0.0015 (± 0.0005) g of fluorescent micronized dust (Radiant) and lightly shaken. Dust of different colours was randomly assigned to the different acclimation treatments, and changed between releases. To release flies, vials with flies from the different acclimation treatments were randomly arranged in a container and foam stoppers were removed from the vials.

Capture points consisting of banana baits in buckets were positioned in a circle around the release point in four directions (north, south, east, and west). Buckets were placed 5–30 m from the release point, for a total of 24 buckets per release. Temperatures were recorded with data loggers (Tinytalk II) placed in the shade 1 m above the ground close to the release site (Table 1). Releases took place in woodland in Victoria, Australia, in a habitat without soft fruit where *Drosophila* populations might breed. The woodlands consisted of eucalyptus or pine trees with flowering shrubs. In the three releases, flies were captured at the buckets at 10.00, 12.00, 14.00, 16.00, and 18.00 h (capture rounds 1, 2, 3, 4, and 5, respectively) (Table 1). Captured flies were held on ice in the field to incapacitate them and minimize transfer of dust colours between the flies. They were then transported to the laboratory and frozen until colours were scored under ultraviolet light (Blak-Ray, Ultra Violet Products, UK).

Fecundity

Altogether, 120 pairs of 2-day-old male and female flies were collected from each acclimation regime and subsequently distributed at random among the four acclimation regimes to test for fecundity using a full factorial design (30 pairs per test temperature per acclimation group). Flies for fecundity assessments were from vials where larval density was controlled during development (50 eggs per vial with 10 mL medium). Flies were placed in vials with medium-filled teaspoons that could easily be replaced. Teaspoons with medium were

renewed every third day on three occasions for the outdoor test regime and the 17°C and variable 12–22°C indoor temperature regimes. For these three test regimes, we obtained data on fecundity during three time windows each spanning 72 h : days 2–5, days 5–8, and days 8–11. Development was faster at a constant 24°C; to count the eggs before larvae began to develop, teaspoons with medium were thus renewed every second day on four occasions. For this test temperature, we obtained data on fecundity during four time windows each spanning 48 h: days 2–4, days 4–6, days 6–8, and days 8–10. After egg-laying, spoons with medium and eggs were frozen for later determination of the number of eggs laid.

Ramping cold knockdown temperature assay

We investigated the critical thermal minima (CT_{min}) under gradual cooling using an ecologically relevant rate of temperature change of $0.1°C \cdot min^{-1}$ (for details and justification, see Overgaard *et al.*, 2012). Briefly, CT_{min} was scored by placing individual flies in empty 5 mL glass vials that were subsequently submerged in a water bath preset at 25°C. The temperature of the water bath was then gradually decreased from 25°C to 0°C at a rate of $0.1°C \cdot min^{-1}$, and CT_{min} was recorded as the temperature at which the flies were knocked down by cold and became unable to move any body part. Subsequently, the flies were maintained at 0°C for 1 h before temperatures were gradually raised again at a rate of $0.1°C \cdot min^{-1}$. During the heating period, we scored the temperature at which the flies regained the ability to stand after being knocked down by the low temperatures ($T_{recovery}$). Approximately 30 female flies were tested from each temperature acclimation treatment group (see Overgaard *et al.*, 2011).

NMR metabolomics

Flies used for the metabolomic investigation were density controlled during development by collecting 50 eggs into eight vials with 10 mL medium per acclimation regime. Emerging flies were quickly sexed under CO_2 anaesthesia and the female flies were returned to their respective acclimation treatments for another 5 days. From each of the acclimation regimes, five replicates of 20 female flies were subsequently snap frozen in liquid nitrogen (this was done in the morning before temperature switched to 'day temperature' in the fluctuating 17°C acclimation regime). Another set of samples was obtained after being exposed to one night of cold exposure at the release site. Thus, along with the flies used for release–capture (see above), we made five replicate vials each with twenty 5- to 6-day-old female flies from each acclimation treatment at 20.00 h. At 08.00 h the next morning, these flies were sampled and snap frozen in liquid nitrogen. Samples were stored at −80°C until further analysis.

Water-soluble metabolites were extracted by homogenization in 500 μL cold 50% acetonitrile using a TissueLyser (Qiagen, Copenhagen, Denmark). Samples were then centrifuged at 10,000 *g* for 10 min and the supernatant was stored at −80°C until ^{1}H NMR analysis. The supernatant was transferred to new tubes, lyophilized, and stored again at −80°C. Immediately before the NMR measurements, samples were rehydrated in 650 μL of 50 mM phosphate buffer (pH 7.4) in D_2O, and 600 μL of the solution was transferred to a 5 mm NMR tube. The buffer contained 50 $mg \cdot L^{-1}$ of the chemical shift reference 3-(trimethylsilyl)-propionic acid-D4, sodium salt (TSP).

The NMR measurements were performed at 25°C on a Bruker Avance-2 500 spectrometer (Bruker Biospin), operating at a ^{1}H frequency of 500.13 MHz. ^{1}H NMR spectra were acquired using a single-90°-pulse experiment with a 40 ms Carr-Purcell-Meiboom-Gill

(CPMG) delay, and water suppression using presaturation. A total of 256 transients of 8 K data points spanning a spectral width of 24 ppm were collected, corresponding to a total experiment time of 10 min. For assignment purposes, a two-dimensional ^{1}H-^{1}H TOCSY spectrum with 80 ms mixing was acquired.

Due to technical problems with the spectrometer, some of our samples could not be used. Thus we investigated four replicates of flies taken from the constant 24°C and the fluctuating 12–22°C acclimation regimes, three replicates from the constant 17°C acclimation regime, and five replicates from the outside acclimation regime. In addition, we investigated three, three, four, and four replicates of flies after prior exposure to cold conditions in the field from the constant 24°C, constant 17°C, fluctuating 12–22°C, and outside acclimation regime, respectively.

Statistical analysis

For the release data, we compared capture rates in each release between female flies from the different acclimation treatments using the chi-square statistic against an expectation of equal numbers being captured from each group. To indicate the relative capture success of flies from the different breeding regimes, we computed relative capture rates comparing the likelihood of capture of, for example, constant 24°C acclimated flies vs. constant 17°C acclimated flies in each of the three releases. Here, a relative capture success of, for example, 1.06 indicates that 6% more 24°C acclimated flies were caught than 17°C acclimated flies.

Non-parametric Wilcoxon rank tests were used to test for an effect of developmental temperature (acclimation regime) on fecundity, as the data were not normally distributed. Tests were performed for each egg-laying period separately, as well as for eggs pooled across all egg-laying periods. Only vials where both the male and the female survived throughout the entire egg-laying period were included in the analysis, resulting in less than 30 replicates per acclimation and test temperature in some cases. Non-parametric Wilcoxon rank tests were also used to test for an effect of developmental temperature (acclimation regime) on CT_{min} and T_{recovery}, as these data were not normally distributed.

NMR spectra were processed using iNMR (www.inmr.net) with 0.5 Hz line broadening. Spectra were referenced to the TSP signal at –0.017 ppm, automatically phased and baseline corrected, and then aligned using icoshift (Savorani *et al.*, 2010). Data reduction was accomplished by dividing the spectra into 0.005 ppm regions (bins). The region around the residual water signal and the high- and low-field ends of the spectrum were removed. The integrals were normalized to total intensity.

Multivariate data analysis was performed with Simca P + 12.0 (Umetrics, Umeå). Principal component analysis (PCA) was carried out on VAST scaled data (Keun *et al.*, 2003). Five significant principal components were assessed by leaving one out in cross-validations (Wold, 1978). The scores from the PCA were subjected to two-way multivariate analysis of variance (MANOVA) for acclimation regime and temperature treatment. PLS discriminant analysis (PLS-DA) was carried out between groups of samples to identify metabolites affected by acclimation regime and by temperature treatment. PLS-DA was performed on UV-scaled data. To interpret the PLS-DA model in terms of metabolite changes, the correlation coefficients (R) between NMR spectral variations and the predicted values were calculated. A cut-off value for R^2 corresponding to $P < 0.05$ with Bonferroni correction for an assumed number of 100 metabolites was calculated from the distribution of R^2 values in 10,000 permutated data sets. The assignments were done based on chemical shifts only

using earlier assignments and spectral databases (Fan, 1996; Malmendal *et al.*, 2006; Cui *et al.*, 2008; Pedersen *et al.*, 2008). No spiking experiments were performed. Relative glucose and maltose concentrations were calculated based on signals at 4.63, 3.52, 3.46, and 3.44 ppm, and 5.40, 3.94, 3.85, 3.63, and 3.51 ppm, respectively.

RESULTS

Releases

Females acclimated at a constant 17°C and 24°C did not differ in their likelihood of reaching the buckets with food (Table 2). Females acclimated at variable temperatures (12–22°C and outside) were caught more frequently than those acclimated at either a constant 17°C or 24°C (Table 1). There was no consistent difference between the variable temperature regimes (12–22°C and outside) in flies' ability to locate the baits with banana (Table 2).

Cold resistance

Flies developed at 17°C (constant or variable) had the lowest $CT_{\mathbf{min}}$ (highest cold resistance), while flies developed at 24°C had the highest $CT_{\mathbf{min}}$ (Fig. 1A). Results from the recovery temperature followed the same overall pattern; flies acclimated at 17°C (constant or variable) had the lowest $T_{\mathbf{recovery}}$ (they regained consciousness at a lower temperature and thus were more cold resistant) compared with flies acclimated at 24°C (Fig. 1B). Thus, in contrast to the patterns in the release experiment, females acclimated at a constant 17°C and constant 24°C differed significantly in their resistance to low temperatures.

Fecundity

Due to the strong effect of period on fecundity, data were analysed separately for each period. Early fecundity was significantly higher for flies acclimated at a constant 24°C regardless of the test temperature at which fecundity was assessed. In later egg-laying periods, the differences between acclimation regimes diminished (Fig. 2). However, due to the differences in the early egg-laying period, total fecundity was higher (although not always statistically significant) in flies acclimated at 24°C than in the flies acclimated at other temperatures during development.

Table 2. Relative capture success and chi-square statistics testing for differences in capture success between acclimation treatments for female flies

	Relative capture success					
Release number	24°C vs. 17°C	24°C vs. 12– 22°C	24°C vs. outside	17°C vs. 12–22°C	17°C vs. outside	12–22°C vs. outside
1	1.06	0.64***	0.65***	0.60***	0.61***	1.00
2	1.08	0.72**	0.75**	0.67***	0.70***	1.04
3	1.08	0.56***	0.78*	0.51***	0.72**	1.41***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

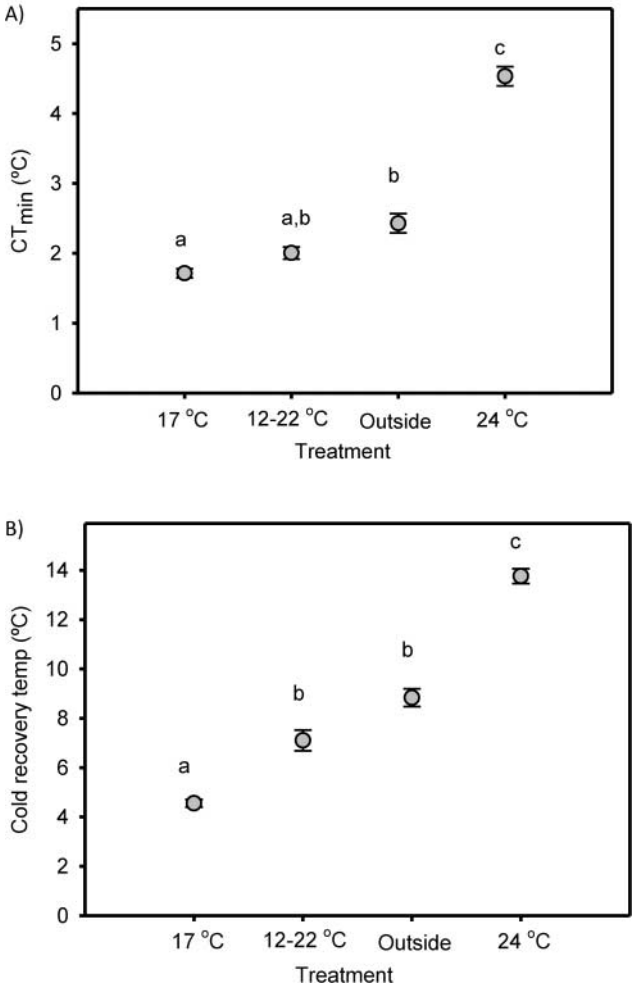

Fig. 1. (A) Critical thermal minima (CT_{min}; ± S.E.) of females from the acclimation treatments (constant 24°C and 17°C, variable 12–22°C, and outside). Flies were tested in a ramping assay where temperatures were gradually decreased from 25°C to 0°C at a rate of 0.1°C · min^{-1}. The temperature at which flies enter a coma is registered. (B) Cold recovery temperature ($T_{recovery}$; ± S.E.) of females from the acclimation treatments (constant 24°C and 17°C, variable 12–22°C, and outside). After gradually decreasing the temperature from 25°C to 0°C at a rate of 0.1°C · min^{-1} and maintaining it at 0°C for 1 h, flies were exposed to an increase in temperature at a rate of 0.1°C · min^{-1}. The temperature at which flies regain the ability to stand on their legs is registered. Different letters denote significant differences ($P < 0.05$) between flies from the different acclimation treatments.

NMR data

Figure 3 shows a typical *D. melanogaster* metabolite ^{1}H NMR spectrum, where the well-resolved metabolite signals have been assigned. The overall relationship between the metabolomic responses of flies to different developmental temperature conditions was assessed by applying principal component analysis (PCA) on the NMR spectra of the

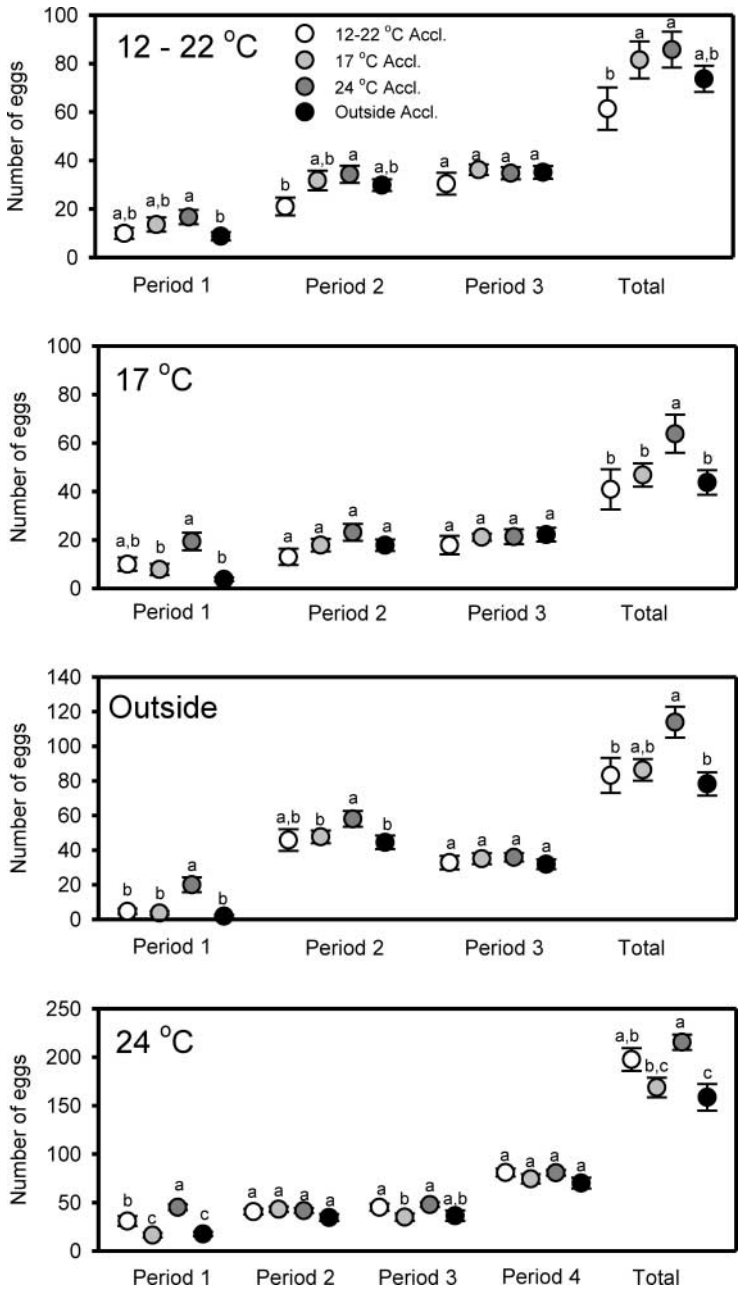

Fig. 2. Fecundity assessed as the number of eggs laid per female (± S.E.) in three or four different time periods or as the total number produced throughout the whole period. Flies acclimated in each of the four different temperature regimes (constant 24°C and 17°C, variable 12–22°C, and outside) were tested under all four thermal conditions. Different letters (within each test period or for the total number of eggs laid throughout the test period) denote significant differences ($P < 0.05$) between flies from the different acclimation treatments.

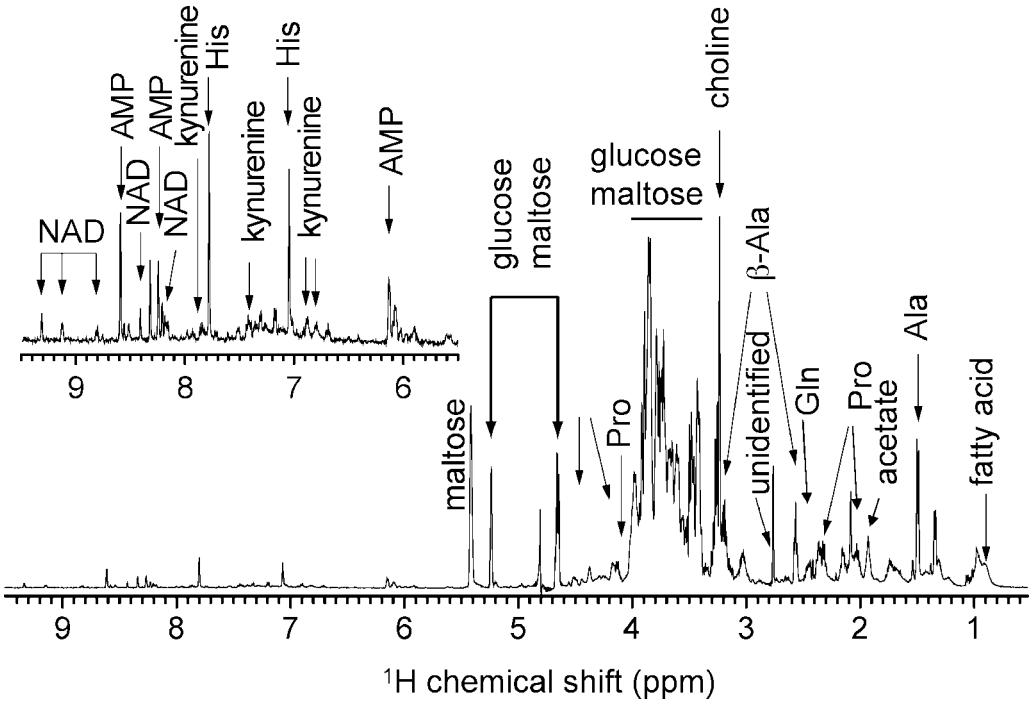

Fig. 3. ^{1}H NMR spectrum of *Drosophila melanogaster* metabolites. Well-resolved signals from some of the most abundant metabolites are assigned. The vertical scale in the insert is five times larger than in the full spectrum.

samples of flies exposed to non-stressful temperatures. The relationship between the metabolite response to developmental temperature and to exposure to low temperature was characterized by PCA (Fig. 4A) and hierarchical cluster analysis (HCA; Fig. 4B) on the NMR spectra of flies sampled directly from the acclimation temperatures or after exposure to low temperatures. As seen from Fig. 4A, the flies developed at a constant 24°C showed a clearly different metabolic profile from the three groups developed at lower temperatures. Furthermore, flies from the constant 24°C acclimation treatment had a different metabolomic response to low temperature exposure compared with the other groups. This was corroborated by results from a two-way MANOVA, which showed significant effects of developmental temperature conditions, exposure temperature, and an interaction between these two factors ($P < 0.0001$). The response to low temperature exposure was therefore dependent on developmental temperatures. The hierarchical cluster analysis (Fig. 4B) also showed that the control and cold-treated constant 24°C acclimated flies differed most from the remaining groups. Untreated flies from the other developmental temperature treatment groups clustered together, as did the cold exposed flies.

Given that flies acclimated at a constant 24°C had a distinct metabolic profile, levels of identifiable metabolites from this treatment were contrasted to levels in flies from the other groups. There was more tyrosine (7.11, 6.89 ppm), kynurenine (7.85, 6.89, 6.80 ppm), UMP (8.10, 5.98, 4.42–4.25 ppm) and potentially other nucleosides or nucleotides, and less glucose (5.22, 4.63, 3.40 ppm) and an unidentified metabolite previously observed in *D. melanogaster* (3.03, 2.74, 2.29, 1.90 ppm) (e.g. Pedersen *et al.*, 2008; Sarup *et al.*, 2012) in the 24°C

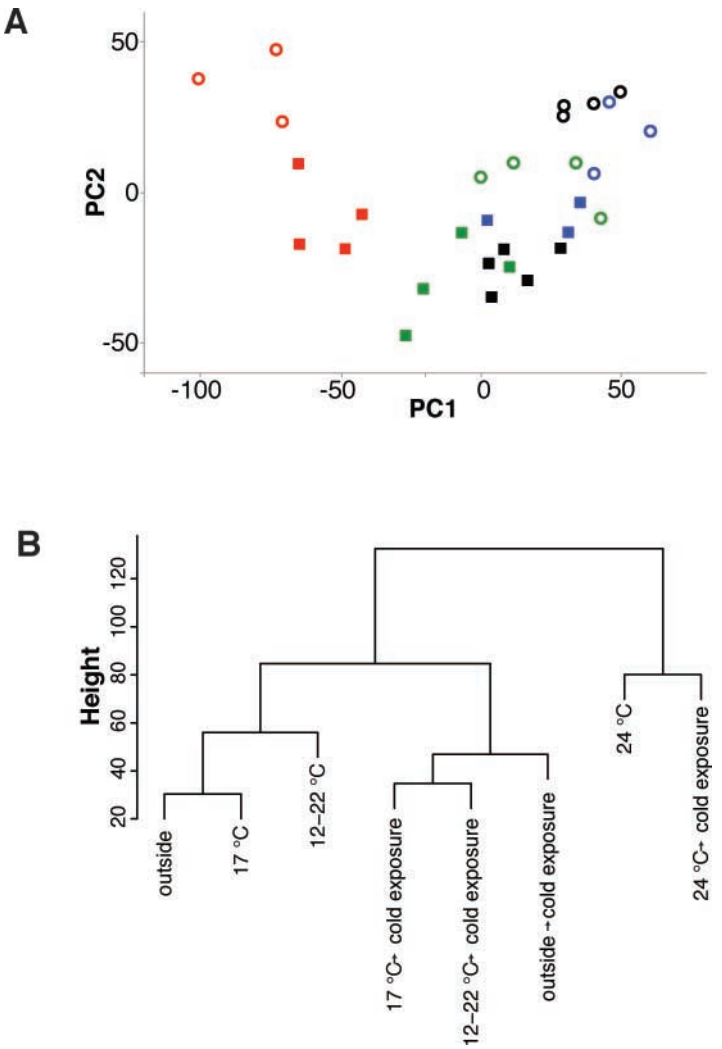

Fig. 4. PCA scores (A) and HCA dendrogram (B) for metabolite samples of flies developed at different developmental temperatures and sampled directly from each of the four acclimation treatments or after prior exposure to cold temperatures in the field. PC1 and PC2 scores: constant 24°C (red symbols), constant 17°C (blue symbols), variable 12–22°C (green symbols), and variable outside (black symbols). Samples collected directly from the developmental acclimation treatments are shown as solid squares whereas those sampled after prior exposure to cold are shown as open circles. PC1 and PC2 explain 40% and 13% of the variation between samples.

acclimated flies. Flies from all three remaining developmental acclimation groups (constant 17°C, 12–22°C, and outside) were also distinguishable ($P < 0.05$).

When the three acclimation treatment groups from a constant 17°C, variable 12–22°C, and outside were exposed to cold temperatures in the field, we observed an increase in glucose (5.22, 4.63, 3.40 ppm) and maltose (5.39, 3.95 ppm), whereas AMP (8.60, 6.13, 4.35 ppm), NAD (9.33, 9.13, 8.81, 6.07, 4.52–4.34 ppm), phenylalanine (7.41–7.32 ppm),

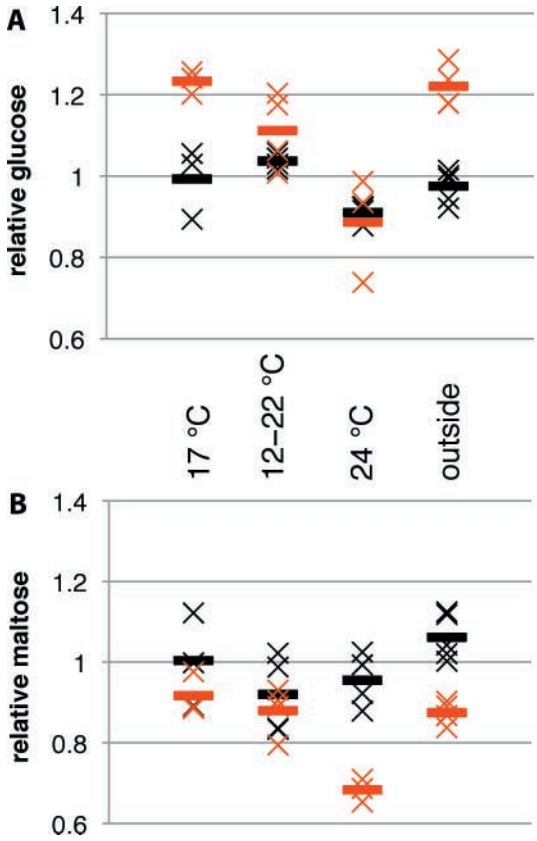

Fig. 5. Relative concentrations of glucose (A) and maltose (B) as calculated from signal intensities. Samples collected directly after the developmental acclimation treatments are shown in black whereas those sampled after exposure to cold conditions are shown in red. The concentrations were calculated relative to the average value in flies sampled from the 17°C acclimation regime without prior exposure to cold temperatures. Concentrations were calculated from spectral areas unique to glucose and maltose.

β-alanine (3.17, 2.54 ppm), glutamine (2.42, 2.13 ppm), and leucine (1.90, 0.93 ppm) decreased. When the constant 24°C developmental treatment group was exposed to the same cold treatment, the only significant change was a decrease in maltose. The relative levels of glucose and maltose in all samples are displayed in Fig. 5.

DISCUSSION

The main aims of the present study were to examine if responses to specific acclimation conditions are consistent across traits, test conditions, and levels of biological organization. Our results show clearly that this is not the case. The effects of acclimation temperature are highly trait and environment specific and differ across levels of biological organization. The effects of different acclimation temperatures therefore depend on whether they are tested in the laboratory or under semi-natural conditions, and both the mean and the variance in developmental and test temperatures affect the acclimation responses. Moreover,

developmental acclimation temperature strongly affects the metabolome. A constant acclimation temperature of 24°C gave rise to a distinct metabolic profile compared with the other acclimation temperatures, which responded differently to temperature shifts.

Development at the highest temperature investigated (24°C) led to poor performance in the release–capture experiments performed at cool temperatures compared with flies developed outside or at a variable 12–22°C (Table 2). However, we observed no difference in the ability to locate resources between flies developed at a constant 17°C and a constant 24°C. Thus, it seems that acclimation at variable cool temperatures improve flies' ability to locate resources. In this context, the results support the beneficial acclimation hypothesis because phenotypic changes induced by acclimation enhance performance of an individual in that particular environment (Levins, 1969; Leroi *et al.*, 1994; Wilson and Franklin, 2002; Kristensen *et al.*, 2008). However, constant 17°C conditions did increase cold resistance (higher $CT_{\mathbf{min}}$ and $T_{\mathbf{recovery}}$) relative to a constant 24°C to the same extent that resistance was increased in the variable cold conditions. Development at lower temperatures generally increased cold resistance in accordance with observations in previous studies (Rako and Hoffmann 2006; Overgaard *et al.*, 2012).

In contrast to the data from the release–capture and cold resistance assays, fecundity showed a completely different pattern, with the females acclimated at a constant 24°C producing more eggs at all test temperatures in the first egg-laying period (Fig. 2). Although this effect diminished in the later egg-laying periods, it nevertheless resulted in higher fecundity overall for this treatment. Since 24°C is considered to be the optimal temperature for *D. melanogaster*, this result is in accordance with the so-called 'optimal developmental temperature' hypothesis, which states that individuals perform best when developed at intermediate temperatures (Huey *et al.*, 1999; Wilson and Franklin, 2002; Steigenga and Fischer, 2009). This pattern runs against expectations based on body size, as smaller animals usually have a lower fecundity (Roff, 1992). In *D. melanogaster*, ovariole number increases with thorax length, although the strength of this relationship depends on nutritional condition (Bergland *et al.*, 2008). Therefore, we expected flies in our warmer acclimation treatment (24°C) to emerge smaller and lay fewer eggs. Although the size of the emerging flies was not quantified, flies acclimated at a constant 24°C were clearly smaller than those emerging from the other acclimation groups. Fecundity was therefore influenced by factors unrelated to size.

The environment-specific and trait-specific costs and benefits of acclimation make it difficult to predict the net fitness outcome of acclimation in natural populations. The benefits of cold acclimation with respect to increased cold resistance and performance in the field detected here and in other studies (Kristensen *et al.*, 2008; Steigenga and Fischer, 2009) may be outweighed by costs associated with lower fecundity. Other studies also suggest that acclimation can have a negative effect on other performance measures. For instance, adult leafminers (*Liriomyza huidobrensis*, Blanchard) exposed to mild heat shocks increase their heat resistance later in life without influencing their cold resistance, but at the same time experience a loss of fecundity (Huang *et al.*, 2007). Similarly, heat-acclimated *D. melanogaster* have increased heat resistance following acclimation but suffer a cost in terms of decreased fecundity under milder conditions and similar costs are found in relation to cold hardening (Krebs and Loeschcke, 1994; Overgaard *et al.*, 2007). The costs of acclimation under field conditions can be much larger than in the laboratory. For example, heat-acclimated *D. melanogaster* are better at locating food resources under warm conditions in the field but this comes at a large cost under milder conditions (Loeschcke and Hoffmann, 2007), while flies that have developed under cold conditions are up to 36 times less likely to locate food in the field in warm conditions than flies reared at intermediate temperatures (Kristensen *et al.*, 2008). Trait- and

environment-specific acclimation responses were also observed in an experiment in which *D. melanogaster* were successfully selected for increased ability to reach a resource at low temperature in the field (Overgaard *et al.*, 2010). Populations that had clearly different abilities to locate resources at cold temperatures in the field were however indistinguishable when tested in standard laboratory tests of cold performance, revealing an irregular relationship between cold performance in the field and in the laboratory [for a general discussion on the ecological relevance of results from laboratory selection experiments, see Harshmann and Hoffmann (1999)].

In line with a recent study by Terblanche *et al.* (2010), we found that temperature variation influences performance in addition to the responses that are associated with changes in mean temperature. In this case, benefits of acclimation at a variable 12–22°C and outside temperatures were detected in the field releases, whereas the constant 17°C acclimated flies did not perform better than the 24°C acclimated flies. These differences were observed despite the rather similar metabolomic profile of the flies experiencing 17°C and the variable temperatures. The metabolomics fingerprint of flies acclimated to 24°C is clearly different from the other three (colder) acclimation regimes (Figs. 3 and 4), and 24°C acclimated flies also display a markedly different metabolomic response to cold exposure. The profile differences only correlate with differences among treatments for the laboratory estimate of cold resistance. The 24°C acclimated flies are characterized by higher levels of nucleosides and nucleotides and a lower level of glucose when sampled directly from the acclimation treatments. After exposure to cold in the field, the flies from the constant 17°C, 12–22°C, and outside conditions increased their levels of glucose whereas maltose, AMP, NAD, phenylalanine, β-alanine, glutamine, and leucine decreased. In contrast, the flies from the constant 24°C developmental treatment group experienced unaltered glucose levels and a significant decrease in maltose. Several previous studies have reported moderate increases in sugars or other compatible osmolytes associated with cold acclimation of chill-sensitive insects (Chen *et al.*, 1987; Yoder *et al.*, 2006; Lalouette *et al.*, 2007; Michaud and Denlinger, 2007; Overgaard *et al.*, 2007). Although the NMR approach used here is mainly qualitative, the magnitude of these changes does not involve the large accumulation of cryoprotectants found in freeze-avoiding or freeze-tolerant insects (Sømme, 1982; Zachariassen, 1985). Moderate increases in sugar levels may, however, help to stabilize membrane function at low temperature by suppressing the phase separation temperature (Mahajan and Tuteja, 2005; Cacela and Hincha, 2006).

In summary, our findings emphasize the notion that developmental acclimation effects are complex, and are dependent on the trait assessed and variability in temperature as well as mean temperature. This makes it challenging to connect results from laboratory experiments to field conditions and to make generalizations about likely benefits and costs of acclimation. Effects of developmental acclimation on the metabolome may link with cold resistance but not necessarily with other measures of performance.

ACKNOWLEDGEMENTS

We are grateful to Jennifer Shirriffs, Carla Sgró, Vanessa Kellermann, Belinda van Heerwarden, and Lea Rako for field collections and help in the laboratory. Funding was provided by the Danish Natural Research Council with a Steno stipend to T.N.K. and a Sapere aude stipend to J.O. A.A.H. was supported by Australian Research Council Fellowship and Discovery grant schemes.

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