

RESEARCH ARTICLE

Critical thermal limits affected differently by developmental and adult thermal fluctuations

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ABSTRACT

Means and variances of the environmental thermal regime play an important role in determining the fitness of terrestrial ectotherms. Adaptive phenotypic responses induced by heterogeneous temperatures have been shown to be mediated by molecular pathways independent of the classic heat shock responses; however, an in-depth understanding of plasticity induced by fluctuating temperatures is still lacking. We investigated high and low temperature acclimation induced by fluctuating thermal regimes at two different mean temperatures, at two different amplitudes of fluctuation and across the developmental and adult life stages of *Drosophila melanogaster*. For developmental acclimation, we found mildly detrimental effects of high-amplitude fluctuations for critical thermal minima, while the critical thermal maxima showed a beneficial response to higher amplitude fluctuations. For adult acclimation involving shifts between fluctuating and constant regimes, cold tolerance was shown to be dictated by developmental temperature conditions irrespective of the adult treatments, while the acquired heat tolerance was readily lost when flies developed at fluctuating temperature were shifted to a constant regime as adults. Interestingly, we also found that the effect of fluctuations at any life stage was gradually lost with prolonged adult maintenance, suggesting a more prominent effect of fluctuations during developmental compared with adult acclimation in *D. melanogaster*.

KEY WORDS: Acclimation, Cold tolerance, *Drosophila melanogaster*, Phenotypic plasticity, Temperature resistance

INTRODUCTION

The role of environmental temperature in regard to organismal survival has long been acknowledged and studied (Bigelow, 1921; Cowles and Bogert, 1944). The strong dependency on temperature explains why organisms have evolved adaptations in behavioural, morphological and physiological traits that enable them to cope with temperatures across natural environments. These adaptations need to accommodate temperature changes on a temporal scale that ranges from between years to within years and/or within days. Adaptation to fluctuating temperatures can affect insect life history and survival differently compared with constant temperature adaptation (Beardmore and Levine, 1963; Long, 1970) and temperature dynamics affect the spatial as well as temporal distribution of species (Bozinovic et al., 2011). This means that adaptive responses induced by fluctuations and our understanding

of them are of interest in their own right, but is also of growing importance in view of the increase in the frequency (Fischer et al., 2010) and severity of thermal extremes that accompany the average environmental temperature changes due to climate change (Gunderson and Stillman, 2015; Bozinovic et al., 2016).

Small ectotherms are thought to be especially affected by the surrounding temperatures because of a limited scope for behavioural and physiological thermoregulation (Overgaard et al., 2011). The responses to fast thermal changes, such as those experienced during night–day cycles or during sudden weather shifts might not effectively be mediated by evolutionary changes in trait means. Instead, phenotypic plasticity (acclimatization) has been proposed as the key mechanism for ensuring the continued physiological performance of ectotherms during thermal fluctuations (Ghalambor et al., 2007; Gerken et al., 2015). From current research, it is evident that species presently surviving at temperatures that are close to their upper thermal limits are challenged in their ability to buffer the effects of climate change via phenotypic plasticity or evolutionary adaptation (Deutsch et al., 2008; Hoffmann et al., 2013). This raises questions as to how well plasticity can further buffer environmental perturbations (Seebacher et al., 2014; Gunderson and Stillman, 2015; Sørensen et al., 2016a). However, the current knowledge of the thermal adaptation and acclimation ability of ectotherms relies for the most part on laboratory investigations at constant temperatures (Niehaus et al., 2012). This has spurred an interest in investigating how well performance in a constant environment reflects performance in a fluctuating environment (Kingsolver et al., 2009; Folguera et al., 2011; Bowden et al., 2014; Estay et al., 2014) and how the molecular mechanisms induced by fluctuations resemble or differ from responses to constant temperature (Podrabsky and Somero, 2004; Sørensen et al., 2016b). The effects of temperature and thermal fluctuations might manifest themselves in numerous life history traits; however, in this study we focus exclusively on critical thermal limits.

Experiments exposing insects to thermal fluctuations have found examples of beneficial high (Bozinovic et al., 2011; Manenti et al., 2014; Sørensen et al., 2016b; but see Overgaard et al., 2011) and low temperature acclimation (Boardman et al., 2013). The outcome of thermal fluctuations probably depends on the exact experimental protocol (Colinet et al., 2015). Here, we focused on diurnal fluctuations with cold dark (night) and warm light (day) periods of varying amplitude (± 4 or 8°C); however, factors such as the number of exposures, duration of each exposure and the product of these also clearly contribute to the final phenotype (Marshall and Sinclair, 2015; Kingsolver et al., 2016). If fluctuations reach the boundaries of the thermal limits, organisms are temporarily stressed and performance can be negatively affected (Deutsch et al., 2008; Huey et al., 2009; Folguera et al., 2011). Alternatively, fluctuations might be considered to relieve organisms from continuous stress and allow for recovery before the next fluctuation (Renault et al., 2004; Boardman et al., 2013). Finally, if temperatures fluctuate within non-stressful limits, they will affect the temperature-controlled metabolism (Estay et al.,

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2014) as well as the assumed costs of monitoring the environment and inducing plastic responses (Murren et al., 2015).

Drosophilids follow linear reaction norms for critical thermal minimum and critical thermal maximum (CT_{min} and CT_{max}) based on mean developmental temperatures (Schou et al., 2016). Thus, a simple trade-off between the high- and low-temperature phase of a fluctuation is expected, with higher temperature acclimation leading to improved heat tolerance and loss of cold tolerance and vice versa. The net outcome of fluctuating thermal acclimation could therefore be hypothesized to be zero (if high and low temperatures experienced during acclimation cancel each other out). Alternatively, fluctuations could lead to more complex acclimation responses for heat and cold tolerance independently, if fluctuations induce alternative mechanisms in response to cold and heat acclimation (Fig. 1). This latter type of response was demonstrated in a winter-acclimatized *Drosophila subobscura* population which had markedly improved cold tolerance without losing upper thermal tolerance as compared with their laboratory-reared conspecifics, possibly as a consequence of the naturally variable thermal conditions (Sørensen et al., 2015).

In order to increase our understanding of the effect of diurnal temperature fluctuations on thermal acclimation ability, we here investigated the relative contribution of means and variances of temperature to heat and cold tolerance acclimation of *Drosophila*

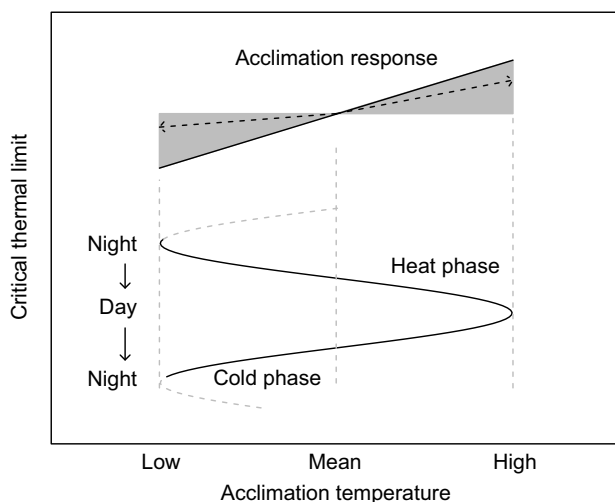


Fig. 1. Conceptual diagram showing the hypothesized effect of fluctuating temperature acclimation relative to constant temperature acclimation. A thermal fluctuation cycle is depicted going from low temperature at night to high temperature during the day and back to low temperature the following night. The straight solid line above the fluctuation indicates a linear acclimation response for constant temperature (both critical minimum and critical maximum), with higher acclimation temperature leading to a gain in heat and a loss in cold resistance and vice versa. The shaded region shows the hypothesized range of acclimation responses from either the heat phase or the cold phase of thermal fluctuations. During the cold or heat phase, the acclimation response is expected to fall between no response (corresponding to acclimation at a constant mean temperature) and the response corresponding to acclimation to a constant low or high temperature, respectively. The net effect of a given treatment will depend on the balance between effects of the heat and cold phases combined. The dashed arrows depict a theoretical example where a fluctuating treatment leads to a small depression of tolerance during the cold phase and a proportionally larger gain during the heat phase. Note that critical thermal minimum and maximum (CT_{min} and CT_{max}) might respond differently to a given treatment. It is uncertain whether increasing temperatures below the mean temperature will lead to heat acclimation (due to the increasing temperature) or cold acclimation (due to the low average temperature). Similar uncertainty applies to the initial part of the cold phase.

melanogaster in a systematic manner. Furthermore, to address the interactions between developmental and adult acclimation responses, we included a full factorial design of developmental and adult acclimation treatments. To investigate the effect of the amplitude of fluctuations, we applied two different regimes with different amplitudes. Based on previous research, we expected both mean and fluctuation of temperature to contribute to thermal acclimation (Bozinovic et al., 2011; Niehaus et al., 2012; Sørensen et al., 2015; Slotsbo et al., 2016). Specifically, we expected that the heat and cold phase of a fluctuation would cancel each other out with respect to cold tolerance acclimation and, thus, that cold tolerance would be determined by the mean acclimation temperature. For heat tolerance, we expected the temperature reached during the heat phase to be responsible for the accumulation of heat tolerance acclimation (Fig. 1). We expected developmental acclimation to be, at least partly, reversible in the adult stage, although not necessarily similarly for heat and cold acclimation (Slotsbo et al., 2016). Finally, we expected any beneficial or detrimental effects of thermal fluctuations to increase with increasing amplitude of the fluctuations.

MATERIALS AND METHODS

Experimental animals

A Danish population of *Drosophila melanogaster* Meigen 1830 collected in 2013 was used for the experiments (Schou et al., 2015). The population was maintained in the laboratory at 25°C (12 h light:12 h dark) for several generations before experimentation. The density of the flies used for experiments was controlled by transferring approximately 40 (± 3) eggs into vials containing 7 ml standard oatmeal–sugar–yeast–agar *Drosophila* food medium. Eggs from different groups of females were randomly divided among vials to avoid several vials of the same treatment group receiving eggs from the same group of females. We used only male flies for the assays to prevent interference from the reproductive status.

Developmental acclimation

Two sets of thermal treatments were applied during development from egg to adult stage. In the first set, the treatments were divided into four groups: 19C (19°C, constant), 19F (19 \pm 4°C, fluctuating), 23C (23°C, constant) and 23F (23 \pm 4°C, fluctuating), i.e. two main treatments (constant and fluctuating) and two main temperatures (19 and 23°C) (Fig. 2A). In the second set, we tested the same treatments, but with the 19F and 23F fluctuating treatments reaching amplitudes of $\pm 8^\circ\text{C}$. In both cases, the fluctuations followed a sinus function across 12 h. Thus, the 19F $\pm 4^\circ\text{C}$ treatment would start at 19°C, increase to 23°C over 6 h and then decrease to 19°C during the following 6 h. For the following 12 h, the temperature would follow a mirrored pattern reaching 15°C and generating a mean temperature of 19°C. A 12 h light:12 h dark photoperiod was used throughout the acclimation period, with light being on during the high temperatures in the fluctuating treatments. Newly emerged flies were sorted under CO₂ anaesthesia and returned to the same treatments for 2 days (recovery time) before being used in heat and cold tolerance assays.

Adult acclimation

To investigate the acclimation effects of exposure of adults to fluctuating temperatures and the reversibility of the developmental acclimation effects (i.e. reversibility of tolerance gained from development upon a change in adult acclimation regime), we exposed adult flies to the thermal acclimation treatments described above. Flies were in all cases treated as described for developmental acclimation, before being transferred to their adult thermal regimes. We investigated the acclimatory effect of thermal fluctuations in

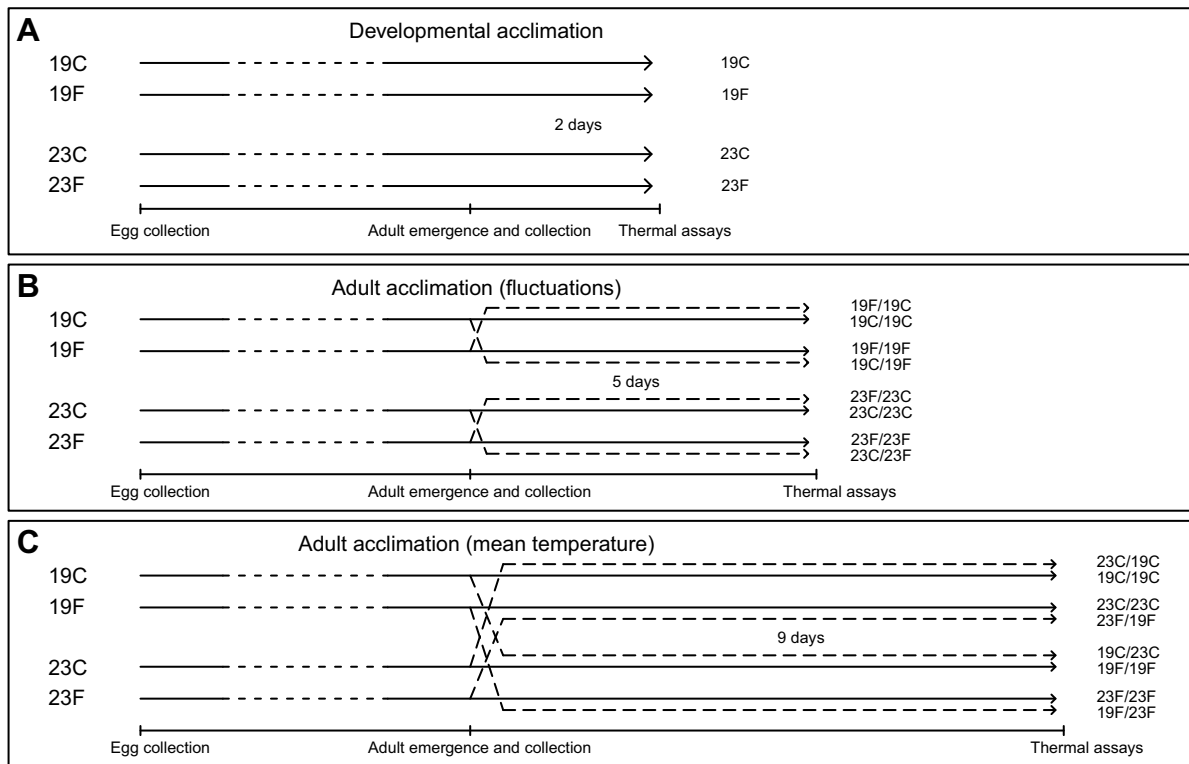


Fig. 2. Experimental regimes used to investigate the effects of mean temperature and thermal variability in developmental and adult life stages of *Drosophila melanogaster*. Thermal treatments were constant 19 or 23°C (19C and 23C, respectively) or fluctuating with a mean of 19 or 23°C (19F and 23F, respectively). Each set of experiments was performed at ± 4 or $\pm 8^\circ\text{C}$, respectively. The three experiments investigated the effects of (A) developmental acclimation, (B) thermal variability during adult acclimation and (C) mean temperature during adult acclimation. Thermal assays were CT_{\min} and CT_{\max} .

adults (i.e. by changing between a constant and a fluctuating temperature and vice versa) for a period of 5 days (Fig. 2B) and of mean temperature in adults (i.e. by changing between a mean of 19 and 23°C and vice versa) for a period of 9 days (Fig. 2C).

Although adult ageing negatively affects several stress tolerance traits of insects (Bowler and Terblanche, 2008), critical thermal limits of *D. melanogaster* seem to be less affected by adult ageing, especially during the first 10 days after eclosion (Slotsbo et al., 2016). Similarly, Sørensen et al. (2015) found no effect of ageing on *D. subobscura* laboratory-acclimated flies at 20°C over the course of 28 days. Thus, using flies at age 5 and 9 days allowed enough time for a strong acclimation response to develop, whilst keeping within the time frame where CT_{\min} and CT_{\max} estimates were not negatively affected by ageing.

Thermal tolerance assays

The thermal acclimation effects of the applied treatments were evaluated by measuring heat and cold tolerance, assayed by an ecologically relevant ramping assay using CT_{\max} and CT_{\min} indices, respectively (Gibert et al., 2001; Macmillan and Sinclair, 2011; Slotsbo et al., 2016). Flies were removed from their respective treatments during the heating phase of fluctuations when the temperature of constant and fluctuating regimes was similar, and individually transferred to small glass vials and immersed in temperature-controlled aquaria set at 21°C (as this was the mean of the thermal acclimation treatments), containing water (for CT_{\max}) or a 1:1 v/v mixture of water and anti-freeze liquid (for CT_{\min}). The temperature was raised or lowered from 21°C at a rate of $0.1^\circ\text{C min}^{-1}$. The activity of the flies was monitored and the temperature at which all movements ceased (after mild tapping with a rod and stimulation with flash light) was recorded as the thermal limit.

Statistical analysis

We analysed CT_{\min} and CT_{\max} of each independent experiment by ANOVA based on linear models. For developmental acclimation, the parameters for analysis included dependent variables CT_{\max} and CT_{\min} , and fixed factors mean temperature (19 or 23°C) and thermal variability treatment (constant or fluctuating). For combinations of developmental and adult acclimation, the fixed factors included were developmental treatment and adult treatment, respectively. Observer effects were initially included as random factors; however, as estimates of this effect were negligible, it was removed from the models and not considered further. The assumptions for running ANOVA were visually investigated on $Q-Q$ and residual plots. Occasional outliers exceeding 2.2 times the interquartile range were removed. All the analyses were performed using the ‘lme4’ package (v.1.1-5) (Bates et al., 2015) in R (R Core Team, 2016). The phenotypic data can be accessed through Dryad (<https://doi.org/10.5061/dryad.25b8f>; Salachan and Sørensen, 2017). We analysed each experiment independently as our experimental design was incapable of distinguishing effects of age from those of independent experimental blocks. However, as discussed above, estimates of CT_{\max} and CT_{\min} seem to be little affected by age in species of *Drosophila* flies within the age range (up to 9 days) used in this study (Sørensen et al., 2015; Slotsbo et al., 2016), suggesting that age had little impact on our results.

RESULTS

Developmental acclimation

Strong acclimation effects of mean developmental temperature could be seen at both amplitudes (± 4 and $\pm 8^\circ\text{C}$) and for both CT_{\min} and CT_{\max} (Table 1, Fig. 3). No effect of fluctuations was observed

Table 1. Results of ANOVA for effects on CT_{min} and CT_{max} of ±4 and ±8°C amplitude fluctuation after developmental acclimation (Fig. 2A)

	CT _{min} (±4°C)	CT _{min} (±8°C)	CT _{max} (±4°C)	CT _{max} (±8°C)
Treatment (<i>F</i> _{d.f.})	1.2 _{1,62}	28.1 _{1,73} ***	0.2 _{1,62}	57.1 _{1,73} ***
Temperature (<i>F</i> _{d.f.})	660.7 _{1,62} ***	263.4 _{1,73} ***	16.2 _{1,62} ***	31.4 _{1,73} ***
Treatment×temperature (<i>F</i> _{d.f.})	0.3 _{1,62}	1.3 _{1,73}	0.2 _{1,62}	0.3 _{1,73}

CT_{min}, critical thermal minimum; CT_{max}, critical thermal maximum. Assays were performed on adults 2 days post-emergence. Treatment represents constant and fluctuating regimes and temperature represents a mean of 19 and 23°C, respectively. ****P*<0.001.

when the amplitude of fluctuations was mild (±4°C), whereas the ±8°C amplitude of fluctuations resulted in highly significant effects on both CT_{min} and CT_{max} scores. For CT_{min}, fluctuations resulted in detrimental effects, wherein flies experienced a loss of CT_{min} acclimation of around 0.3–0.4°C in their CT_{min} scores compared with those of the constant temperature flies (Fig. 3D). For CT_{max}, beneficial acclimatory effects of high-amplitude fluctuations were observed, with a gain of CT_{max} acclimation of around 0.3–0.4°C in response to fluctuations (Fig. 3B). As there were no significant interaction effects between the factors, these effects were consistent across acclimation temperatures (Table 1).

Effect of fluctuations in the adult stage

Changing the thermal treatments (from a constant to a fluctuating diurnal regime and vice versa) after emergence generally showed small and inconsistent effects for the ±4°C amplitude regime. Significant interactions between developmental and adult thermal regimes for CT_{min} at 19°C (Table 2, Fig. 4C) and for CT_{max} at 23°C (Table 2, Fig. 5A) were the only significant effects. However, with an increase in the amplitude to ±8°C, significant effects of developmental and adult treatments could be seen, but with no interaction effects (Table 2). For CT_{min} developmental fluctuations continued to have a significant detrimental effect at both mean temperatures (Table 2,

Figs 4D and 5D), and at a mean of 19°C, adult fluctuations also contributed with a further detrimental effect (Fig. 4D). The mean fluctuation temperature of 19 and 23°C led to a CT_{min} loss of ~0.3–0.4°C and ~0.5–0.6°C, respectively (Figs 4D and 5D). A minor beneficial effect of adult fluctuations corresponding to ~0.15–0.2°C improvement of CT_{max} was the only significant effect on this trait of the ±8°C amplitude fluctuations (Fig. 5B).

Effects of adult mean temperature

Flies subjected to temperature shifts during the adult acclimation period showed highly significant acclimation effects on their measured CT_{min} scores (Table 3, Figs 6 and 7). Fluctuating regimes in the ±4°C amplitude groups did not show any further significant effect. For the ±8°C amplitude groups, we additionally observed significant effects of the developmental acclimation treatment (with fluctuations during development adding an additional detrimental effect to CT_{min}) and of the interaction between developmental and adult acclimation treatment (Table 3, Figs 6D and 7D). For CT_{max}, changes in temperature alone showed only minor effects. Shifts from 23 to 19°C led to a significant loss of high-temperature tolerance in the ±4°C amplitude experiment (Table 3, Fig. 7A). No effect was observed in the ±8°C amplitude experiment (Fig. 7B). In the opposite shift (from 19 to 23°C), no significant effect of adult treatment was detected. Instead, a significant effect of developmental treatment and an interaction effect between developmental treatment and adult temperature could be seen for the ±8°C amplitude groups (Table 3, Fig. 6B).

DISCUSSION

Developmental acclimation

It is known that increasing the mean temperature results in beneficial heat acclimation and decreasing the mean temperature results in beneficial cold acclimation (Schou et al., 2016; Sørensen et al., 2016a). Hence, the acclimation effects seen between flies incubated at 19 and 23°C in our study are as expected. In contrast, the ±4°C amplitude fluctuation seems to be too low to elicit any major acclimatory effect on either of the critical thermal limits, and hence our discussion from this point will concentrate on the effects of the larger amplitude fluctuation (±8°C).

While constant temperature reaction norms follow linear performance curves in regard to the critical thermal limits (Schou et al., 2016), reaction norms for fluctuating temperatures are multidimensional, depending on amplitude, rate of change and periodicity in addition to temperature (Colinet et al., 2015). Thus, extrapolating the effects of fluctuations based on constant temperature reaction norms should be done with caution (Niehaus et al., 2012). The negative impact on CT_{min} of ±8°C amplitude fluctuations corresponded to a loss of CT_{min} acclimation of around 0.3–0.4°C. In terms of constant temperature acclimation, this would roughly translate to a response of only a 1°C change in temperature during developmental acclimation at constant temperature (Sørensen et al., 2016a). Thus, even if significant effects of fluctuations were observed, their contribution would be minor compared with the effects of the

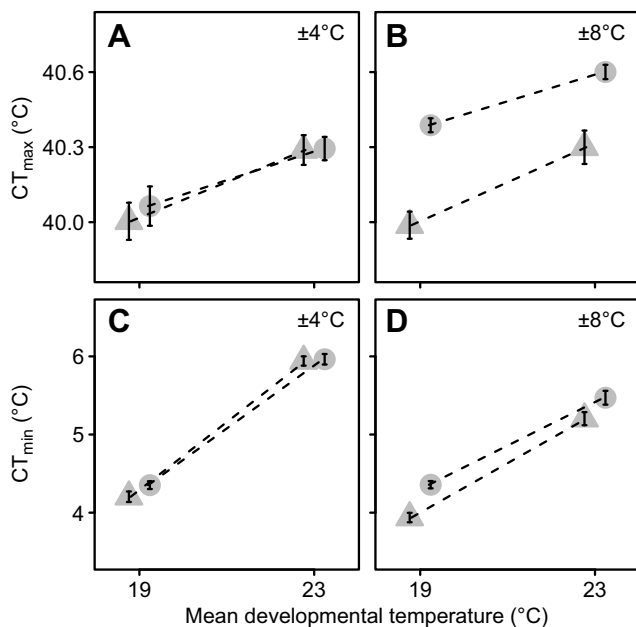


Fig. 3. Critical thermal limits for treatment groups at constant and fluctuating temperatures assayed 2 days after emergence and plotted as reaction norms. (A,B) CT_{max} estimates at ±4°C (*N*=14, 14, 19, 20) and ±8°C (*N*=20, 20, 20, 19), respectively. (C,D) CT_{min} estimates at ±4°C (*N*=14, 14, 20, 20) and ±8°C (*N*=20, 19, 20, 20), respectively. *N* values correspond to treatments 19C, 19F, 23C and 23F, respectively. Data are means±s.e.m. Shaded circles indicate fluctuating treatment and shaded triangles indicate constant temperature treatment.

Table 2. Results of ANOVA for effects on CT_{min} and CT_{max} of ± 4 and $\pm 8^\circ\text{C}$ amplitude fluctuation after adult acclimation (fluctuation shifts, Fig. 2B)

	CT_{min} ($\pm 4^\circ\text{C}$)		CT_{min} ($\pm 8^\circ\text{C}$)		CT_{max} ($\pm 4^\circ\text{C}$)		CT_{max} ($\pm 8^\circ\text{C}$)	
	D19	D23	D19	D23	D19	D23	D19	D23
Developmental treatment ($F_{d.f.}$)	1.9 _{1,33}	0.5 _{1,33}	11.8 _{1,70} **	34.5 _{1,34} ***	0.2 _{1,34}	2.8 _{1,35}	1.8 _{1,73}	0.1 _{1,33}
Adult treatment ($F_{d.f.}$)	0.01 _{1,33}	0.6 _{1,33}	4.1 _{1,70} *	0.6 _{1,34}	0.1 _{1,34}	1.6 _{1,35}	2.3 _{1,73}	7.2 _{1,33} *
Developmental treatment*adult treatment ($F_{d.f.}$)	6.3 _{1,33} *	0.1 _{1,33}	1.3 _{1,70}	1.5 _{1,34}	0.1 _{1,34}	8.2 _{1,35} **	0.5 _{1,73}	1.9 _{1,33}

Assays were performed 5 days post-emergence. The flies used had a mean developmental temperature of 19°C (D19) or 23°C (D23) and were shifted after emergence between constant and fluctuating regimes (see Fig. 2). Developmental treatment and adult treatment represent constant and fluctuating regimes administered during the developmental and adult period, respectively. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

mean temperature acclimation. The beneficial impact of higher amplitude fluctuations on CT_{max} could be a result of the temperature reaching stressful limits and thereby inducing heat stress responses (Lindquist, 1986). In this case, we would expect a much stronger response for the 23F treatment (which reaches 31°C) than for the 19F treatment (which only reaches 27°C). However, our results did not support this notion. Sørensen et al. (2016b) found a similar discrepancy between the responses to fluctuation at mean temperatures of 15 and 25°C , wherein the 15°C fluctuating treatment had a stronger impact on thermal tolerance. This might point to an important role for the amplitude of variation in determining the response to fluctuations.

The detrimental effect of fluctuations on CT_{min} and beneficial effects on CT_{max} suggest that the minimum and maximum temperatures reached during fluctuation affected these two traits differently. This supports our hypothesis that CT_{max} and CT_{min} are independently regulated based on mechanisms that were activated in response to the heat phase and cold phase of fluctuations (see Fig. 1). Whilst the absolute changes in tolerance for both high- and low-temperature critical thermal limits in our study were very similar in absolute terms (0.3 – 0.4°C), as a result of the much steeper

acclimation reaction norm for CT_{min} (Schou et al., 2016; Sørensen et al., 2016a), the response for CT_{max} corresponds to a much larger effect for CT_{max} in terms of constant temperature developmental acclimation equivalents. This, in theory, would make the 19F treatment surpass the CT_{max} of the 23C treatment (note that the 19F treatment fluctuated to a maximum of 27°C and a minimum of 11°C), although, as discussed above, caution is warranted when extrapolating from constant temperature reaction norms. Therefore, we conclude that mean temperature largely determined the CT_{min} acclimation response while some aspects of fluctuation played a more prominent role for CT_{max} acclimation and could even surpass the effects of the mean temperature acclimation response.

Effect of fluctuations in the adult stage

For CT_{min} , the detrimental effect of development at fluctuating temperatures was not matched by any detrimental effect of fluctuation during adult acclimation. Kristensen et al. (2008) also suggested a much more severe response to cold acclimation arising from developmental acclimation than adult acclimation. Constant temperature shifts were readily influenced by adult acclimation temperatures and the effects of developmental temperature were

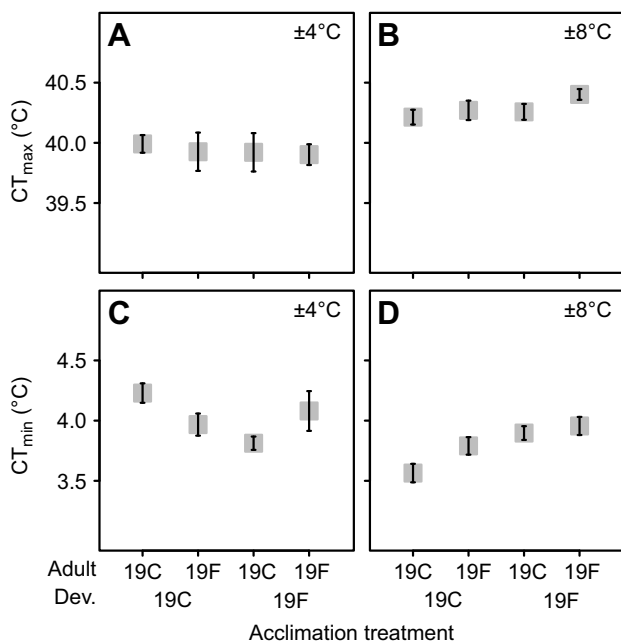


Fig. 4. Critical thermal limits for treatment groups (mean temperature of 19°C) assayed 5 days after emergence. The flies were exposed to a full factorial combination of constant (19C) or fluctuating (19F) developmental acclimation, followed by constant (19C) or fluctuating (19F) adult acclimation (see Fig. 2). (A,B) CT_{max} estimates at $\pm 4^\circ\text{C}$ ($N=10, 10, 8, 10$) and $\pm 8^\circ\text{C}$ ($N=20, 20, 19, 20$), respectively. (C,D) CT_{min} estimates at $\pm 4^\circ\text{C}$ ($N=9, 10, 9, 9$) and $\pm 8^\circ\text{C}$ ($N=20, 20, 16, 20$), respectively. N values correspond to treatment groups from left to right in each panel. Data are means \pm s.e.m.

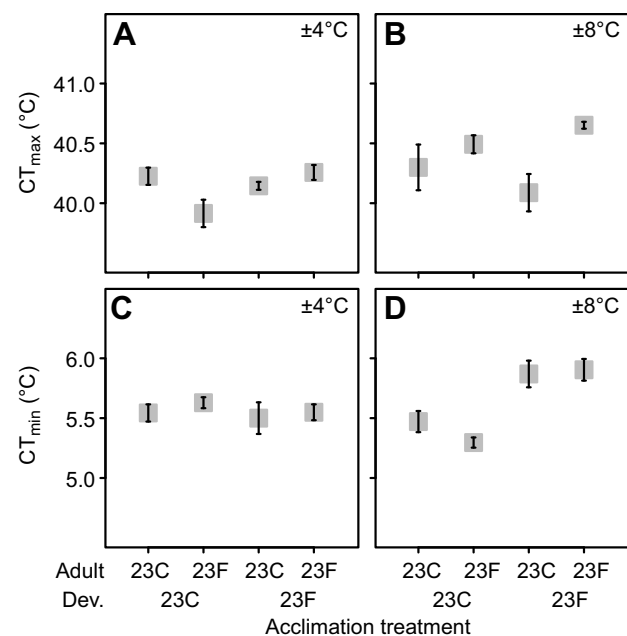


Fig. 5. Critical thermal limits for treatment groups (mean temperature of 23°C) assayed 5 days after emergence. The flies were exposed to a full factorial combination of constant (23C) or fluctuating (23F) developmental acclimation, followed by constant (23C) or fluctuating (23F) adult acclimation (see Fig. 2). (A,B) CT_{max} estimates at $\pm 4^\circ\text{C}$ ($N=10$) and $\pm 8^\circ\text{C}$ ($N=10$), respectively. (C,D) CT_{min} estimates at $\pm 4^\circ\text{C}$ ($N=9, 10, 9, 10$) and $\pm 8^\circ\text{C}$ ($N=9, 10, 10, 10$), respectively. N values correspond to treatment groups from left to right in each panel. Data are means \pm s.e.m.

Table 3. Results of ANOVA for effects on CT_{min} and CT_{max} of ± 4 and $\pm 8^\circ\text{C}$ amplitude fluctuation after adult acclimation (mean temperature shifts, Fig. 2C)

	CT_{min} ($\pm 4^\circ\text{C}$)		CT_{min} ($\pm 8^\circ\text{C}$)		CT_{max} ($\pm 4^\circ\text{C}$)		CT_{max} ($\pm 8^\circ\text{C}$)	
	D19	D23	D19	D23	D19	D23	D19	D23
Developmental treatment ($F_{d.f.}$)	2.8 _{1,35}	5.3 _{1,73}	30.1 _{1,56} ***	10.4 _{1,36} **	0.7 _{1,34}	1.7 _{1,35}	5.7 _{1,56} *	3.3 _{1,34}
Adult temperature ($F_{d.f.}$)	170.0 _{1,35} ***	155.1 _{1,73} ***	94.0 _{1,56} ***	50.7 _{1,36} ***	2.6 _{1,34}	10.8 _{1,35} **	0.7 _{1,56}	0.3 _{1,34}
Developmental treatment*adult temperature ($F_{d.f.}$)	3.7 _{1,35}	0.1 _{1,73}	10.5 _{1,56} **	4.6 _{1,36} *	5.2 _{1,34}	2.3 _{1,35}	4.4 _{1,56} *	0.2 _{1,34}

Assays were performed 9 days post-emergence for the flies developed at a mean temperature of 19°C (D19) or 23°C (D23) and shifted post-emergence between mean adult temperatures of 19 and 23°C (within the same constant and fluctuating regimes). Developmental treatment represents constant and fluctuating regimes during development and adult temperature represents a mean adult temperature of 19 and 23°C . *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

found to be highly reversible (Slotsbo et al., 2016); additionally, the effect of the mean difference in the temperature far surpassed any effect of fluctuations. CT_{max} , in contrast, was more strongly influenced by adult treatment, and acclimation developed during the developmental stage was readily reversed by the adult treatment. In contrast to fluctuating regimes, constant temperature shifts resulted in asymmetrical responses where either an effect of developmental acclimation or no effect of developmental acclimation could be seen (Slotsbo et al., 2016). Regardless, as constant temperature acclimation and fluctuations result in the activation of independent gene sets, it is likely that the final acclimatory outcome would be determined by a complex interplay between developmental and adult temperatures (Sørensen et al., 2016b).

Effects of adult mean temperature

Adults showed expected acclimation responses to mean temperature similar to the responses observed for developmental acclimation, and only minor effects of fluctuations of $\pm 4^\circ\text{C}$ and slightly more prominent effects at $\pm 8^\circ\text{C}$ amplitude (over the experimental adult period of 9 days). Thus, moving mean temperature drove the main

response, weakening the effects of fluctuations. Moreover, stronger effects of 8°C amplitude fluctuations were noticeable for the shift from 23°C to 19°C . Larger effects at larger amplitudes are not surprising and corroborate other studies, e.g. for newt larvae where larger diel fluctuations during embryonic development resulted in faster swimming speed compared with that of newts developed at lower or moderate fluctuating temperatures (Měráková and Gvoždík, 2009). Terblanche et al. (2010) also detected similar enhanced effect of larger diel fluctuations on some of the stress tolerance traits tested, especially on the critical thermal limits in *Ceratitis capitata*. The stronger impact of the larger fluctuations in our study could be a result of the fluctuations entering a more stressful temperature region (19°F fluctuates to a minimum of 11°C) as opposed to the benign temperature conditions of $\pm 4^\circ\text{C}$ amplitude (here, 19°F fluctuates only to a minimum of 15°C). It could then be argued that as colder temperature acclimation results in beneficial acclimation, a temperature of 11°C reached during fluctuation should also elicit such a response. However, because the fluctuations also reach a maximum temperature of 27°C (for 19°F with $\pm 8^\circ\text{C}$ amplitude fluctuations), the final acclimatory outcome is

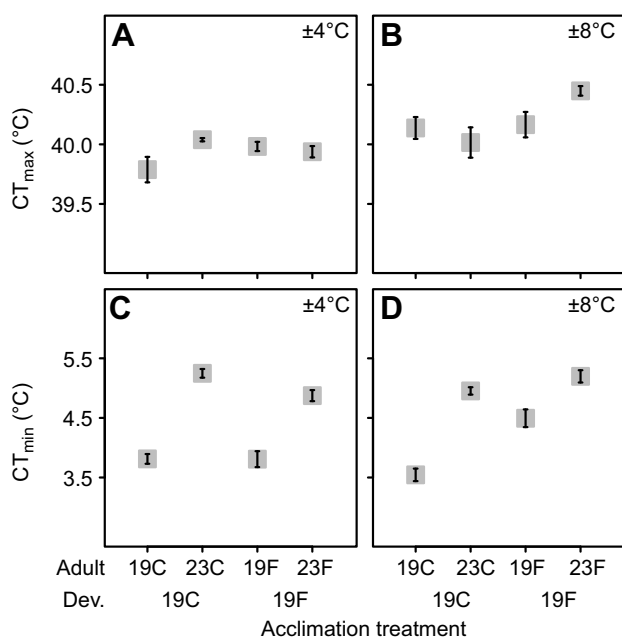


Fig. 6. Critical thermal limits for treatment groups assayed 9 days after emergence (mean developmental temperature of 19°C). The flies were subjected to a combination of mean temperature exposures within the thermal regime, i.e. constant (19°C or 23°C) developmental acclimation, followed by constant (19°C or 23°C) adult acclimation, or fluctuating (19°F or 23°F) developmental acclimation, followed by fluctuating (19°F or 23°F) adult acclimation (see Fig. 2). (A,B) CT_{max} estimates at ± 4 ($N=10$) and $\pm 8^\circ\text{C}$ ($N=15$), respectively. (C,D) CT_{min} estimates at ± 4 ($N=19, 20, 19, 20$) and $\pm 8^\circ\text{C}$ ($N=10$), respectively. N values correspond to treatment groups from left to right in each panel. Data are means \pm s.e.m.

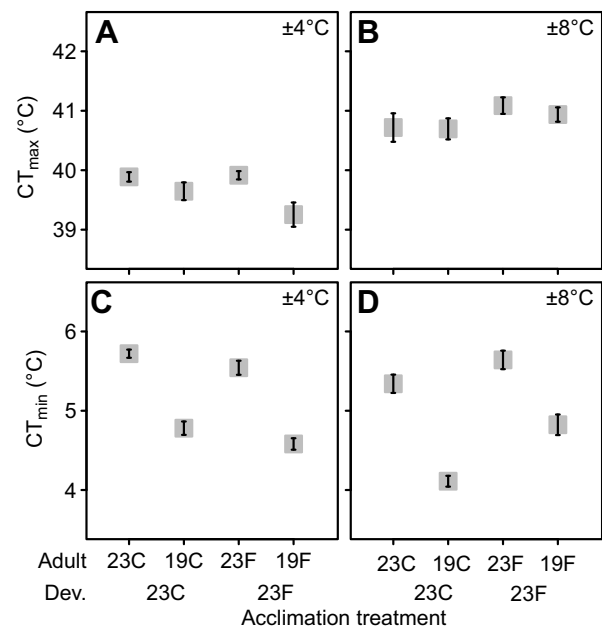


Fig. 7. Critical thermal limits for treatment groups assayed 9 days after emergence (mean developmental temperature of 23°C). The flies were subjected to a combination of mean temperature exposures within the thermal regime, i.e. constant (23°C) developmental acclimation, followed by constant (19°C or 23°C) adult acclimation, or fluctuating (23°F) developmental acclimation, followed by fluctuating (19°F or 23°F) adult acclimation (see Fig. 2). (A,B) CT_{max} estimates at ± 4 ($N=10$) and $\pm 8^\circ\text{C}$ ($N=10$), respectively. (C,D) CT_{min} estimates at ± 4 ($N=19, 20, 19, 20$) and $\pm 8^\circ\text{C}$ ($N=10$), respectively. N values correspond to treatment groups from left to right in each panel. Data are means \pm s.e.m.

more likely to be a result of the interaction between the heat and cold phases of fluctuations.

Do thermal fluctuations lead to important modulation of thermal limits?

The introduction of thermal fluctuations might modulate the linear reaction norms seen for constant developmental temperature acclimation (Niehaus et al., 2012; Schou et al., 2016), dependent on mean temperature, and the amplitude and frequency of fluctuations. For example, Kingsolver et al. (2016) have shown beneficial effects of repeated heat exposure on heat tolerance. Our results have shown that temperature fluctuations affect thermal acclimation responses independent of constant temperature reaction norms and with a different impact in developmental and adult life stages. CT_{\min} was more strongly affected by the mean developmental temperature whereas CT_{\max} was beneficially affected by the higher amplitudes, with the effect surpassing that of mean temperature acclimation. Thus, thermal fluctuations could be an important modulator of CT_{\max} . We used male flies to avoid any potential confounding effects of reproductive status. However, the sensitivity of males and females might differ, which could be addressed in future studies.

The slight detrimental effect of developmental fluctuations on CT_{\min} was maintained with no sign of reversibility during the adult stage when shifted to a constant temperature. However, for CT_{\max} , the effects were slowly reversible during the adult acclimation period. Similar to developmental acclimation, higher amplitude fluctuations had increased effects during adult acclimation, with mean temperature mainly determining CT_{\min} and beneficial acclimation affecting CT_{\max} . With prolonged adult acclimation, the differences between treatments converged, suggesting that fluctuations had less of an effect in the adult than in the developmental life stages. This difference between developmental and adult stages suggest that these life stages rely on different strategies for coping with thermally heterogeneous environments, with the adults potentially being more capable of behavioural avoidance (Krebs and Loeschcke, 1995; Hoffmann et al., 2003). Thus, fluctuating temperatures as applied in this study might be most important for modulating high-temperature hardiness in developmental stages of *D. melanogaster*.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: P.V.S., J.G.S.; Methodology: P.V.S., J.G.S.; Formal analysis: P.V.S., J.G.S.; Investigation: P.V.S., J.G.S.; Resources: J.G.S.; Writing - original draft: P.V.S.; Writing - review & editing: J.G.S.; Visualization: P.V.S.; Supervision: J.G.S.; Funding acquisition: J.G.S.

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Data availability

Data have been deposited in the Dryad digital repository: <https://doi.org/10.5061/dryad.25b8f>

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